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ON ANTIBIOTIC EFFECTS OF LICHENS AND LICHEN SUBSTANCES

BY

K. O. VARTIA

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ON ANTIBIOTIC EFFECTS OF LICHENS AND LICHEN SUBSTANCES

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CORRIGENDA

P 22, line 15.	For »affect» read »effect».
P 44, Table V.	In column Subtilis, for »++» read »+++».
P 46, line 10.	The first to lines from P. 47 should be viserted here above Fig. 5.
P 47, line 6.	For symbol »=» substitute symbol »—».
P 47, Table VIII.	In column Subtilis, for the last five »++» read »+++».
P 59, Table XVI.	In column Dipht. —1:160 000 for »+» read »++».
P 66, line 13.	For »nhibitory» read »inhibitory».

Preface

The medicinal use of lichens has a long history: apart from their ancient uses they have held a certain position among the official drugs of the pharmacopoeias of different countries. Investigation of the chemical components of lichens — primarily of the so-called lichen substances, less appropriately termed lichen acids — had already led to results in the last century. Against this background the modern — antibiotic — study of lichens seems of recent date; the first preliminary antibiotic experiments with lichens were effected as late as 1944—1945 (Burkholder), and by the time the present study was started, January 1948, publications dealing with the antibiotics in lichens totalled some five (further Barry, Marshak, Stoll, Bustinza). In the last two years lichen substances have attracted increasing attention, in the first place owing to their inhibitory effect on the growth of tubercle bacillus (especially Marshak, Shibata, etc.). To date, however, relatively few lichen substances have been investigated in detail. As regards theory, at least, lichen substances, a group of antibiotics of well known chemical composition, are likely to arouse further interest.

* * *

To my teacher, the Chief of the Helsinki University Sero-bacteriological Institute, Professor K. O. Renkonen, I am indebted for the inspiring interest he has shown in my work ever since its inception, and for the great good advice he has given me, untiringly and readily, in the course of its progress. I also wish to thank, in particular, University Lecturer Risto Pätiälä, M.D., and the other researchers and staff of the Sero-bacteriological Institute for the support I have received in my work. To Emeritus Professor Ernst Häyrén I am indebted for the identification of the main part

ot the lichen material and for his decisive guidance and personal participation in its collection. Further, I wish to thank Dr. Veli Räsänen, Ph.D., and Mr. Lars Fagerström, Mag.Phil., for the lichen species given to me, the former also for the identification of most of the Usnea species and numerous other lichens collected by me.

Assistant Professor at the Institute of Chemistry, University Lecturer Salli Eskola, Ph.D., has always been prepared, never grudging her time, to give important advice on the isolation of lichen substances; she also gave me for my experiments anilide of pulvic acid synthesised at the Institute of Chemistry; for all this I wish to express my deep gratitude. To Dr. Vincent C. Barry, Department of Chemistry, University College, Dublin, I am grateful for the Diploicin he sent me; I could not have obtained it in this country. I am also grateful to Mr. Paavo Kajanne, Chemical Engineer, Demonstrator of the Institute of Technology Laboratory of Chemistry, who prepared decarbousnic acid, usnolic acid and atranol for my work. In this connection I also wish to thank everybody else whose peace I have disturbed and whose time I have taken up with my investigation.

Further, I am greatly indebted to Miss Toive Pietarinen, Mag.Phil., for excellent chemical co-operation, and to Miss Ruth Carlstedt, who without counting the hours has assisted in the technical bacteriological work.

Finally I wish to thank Mrs. Hilkka Mäkinen, M.A., and Mr. L. A. Keyworth, M.A. (Cantab.), for the translation of my paper into English.

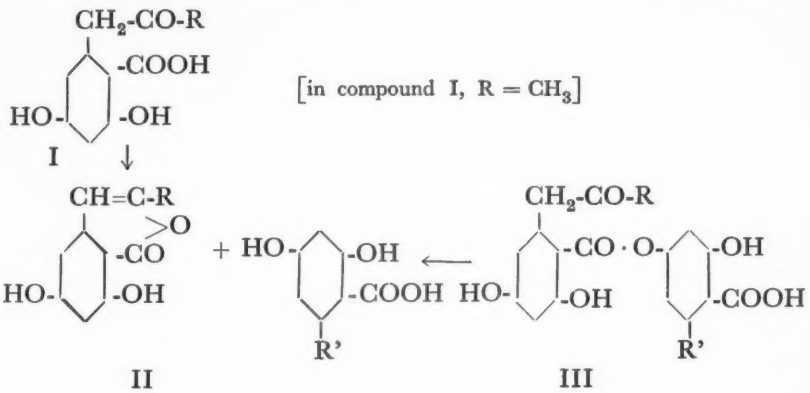
I also gratefully acknowledge the grant received in the course of my work from the Emil Aaltonen Foundation.

Helsinki, August 13, 1950.

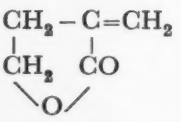
The Author.

Lichens and Lichen Substances

Lichens (Lichenes) have been paid lively attention by the chemists. The majority of the lichens have been found to produce, as a result of their metabolism, so-called lichen substances or lichen acids, organic compounds peculiar to lichens only; attempts to find identical compounds in other vegetable organisms have failed, though colouring matters occurring in lichens have also been discovered elsewhere (81). A fungal colouring matter, atromentin, has also been found to change into dioxypulvic acid on oxidation (46). From some fungi compounds identical with the derivatives or decomposition results of lichen substances have been found: the fungus Sparassis ramosa contains methylester of evernic acid (Sparassol) (3); similarly, the fungus Penicillium brevi-compactum, in sugar medium, produces a compound (I) of which the readily resulting enol-lactone (II) is homologous with the compound resulting from the saponification of any lichen acid of the olivetoric acid type (III) (55):



The compound contained in the fungus Erythronium americanum (IV) is also close in structure to the aliphatic lichen acids of the lactone type (V) (16), which can be considered as its derivatives:



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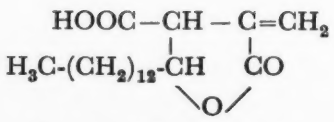


V (Protolichesteric acid)

The denomination »lichen acid» was first employed by Pfaff, 1826, when he found »eine eigentümliche Säure» from Iceland moss (Cetraria islandica) (60). Pfaff's finding, evidently fumarprotocetraric acid (28), was the beginning of the rapidly expanding lichen chemistry. Heeren (26) in 1830, discovered aliphatic roccellic acid, Schunck (79) in 1842 lecanoric acid, Bebert (12) in 1831—1832 vulpic acid, and Rochleder and Heldt (76) and Knop (37), 1843—1844, usnic acid. Knop and Schnedermann demonstrated lichesteric acid in 1845 (38), and stictic acid the year after (39), Stenhouse, 1848, evernic (86) and in 1849 gyrophoric acid (87). Paterno was the first to demonstrate zeorin 1876 (56), atranorin 1877 (58) and rangiformic acid 1882 (57). The largest number of lichen substances has been isolated by Hesse (28—31) and Zopf (96—97), and as a result of their investigations they totalled nearly one hundred and fifty in number by 1907. Of this total, the structure of a few only had been studied; several of them, later, have been proved to be mutually identical or impure etc.

Structural research proper into lichen substances was started by Fischer, who in 1913, both analytically and synthetically, explained the structure of lecanoric acid (21). Spiegel had already given the structural formula of vulpic acid in 1880—1881 (85), and Volhard had also succeeded in synthesising this acid in 1894 (92), but its complete structure was only given in 1926 by Karrer *et al.* (35). Pfau explained in detail the structure of atranorin in 1928 (61), Koller and Krakauer that of cetraric acid in 1929 (42). On the structure of usnic acid no unanimity has yet been reached; the formula given by Widman (93) in 1900 was proved incorrect by Schöpf and Heuck in 1927 (80) and later by Curd, Robertson, Foster and Healy (18).

The decisive work in the study of the structure of lichen substances has been carried out by Asahina and co-workers (2—10). Largely thanks to their investigations, the number of lichen substances thoroughly studied at the moment is about sixty. Mainly

on the basis of the classification worked out by Zopf at the beginning of the century, modestly termed by him as a »temporary one, to provide a general picture» (96), Asahina, in 1934 (2), gave the general systematics of known lichen substances:

A. Aliphatic and alicyclic compounds

1. Fatty acids and lactones

   An example of the former is roccellic acid (I), of the latter lichesteric acid (II). In addition, the group comprises nephromopsinic acid, nephrosterinic and nephrosteranic acids, as well as caperatic and rangiformic acids.

2. The Zeorin Group, neutral compounds not saponifiable by alkalis, the formulae of which are not known, are probably terpene derivatives. Of them, zeorin and leucotylin are known.

3. Sugar alcohols

B. Aromatic compounds

4. Pulvic acid derivatives

   Example: vulpic acid (III). Other pulvic acid derivatives: pulvic acid, pinastric acid, calycin, rhizocarpic acid.

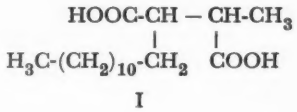
5. Cumarone derivatives

   The only representative, usnic acid (IV) with its derivatives

6. The Thiophene Group

7. Depsides

   Depsides are divided by Asahina into those of orcinol and β-orcinol type. Examples of the former are lecanoric (V) and gyrophoric acids (VI), of the latter atranorin (VII). Evernic acid, mentioned above, belongs to the former type. At present, 27 depsides are known.



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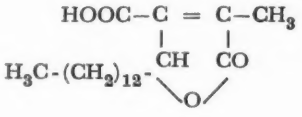

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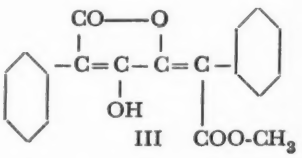

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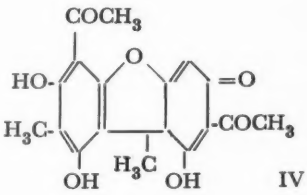

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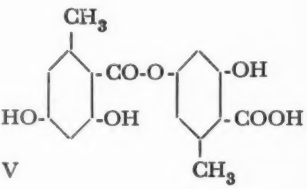

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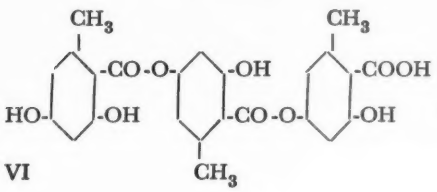
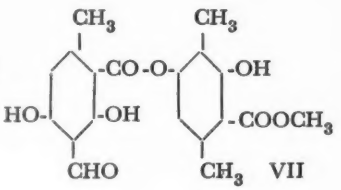


VI



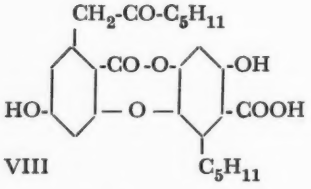
VII

8. Depsidones

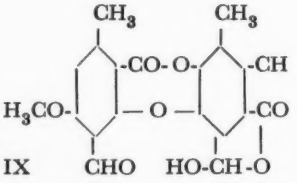
are divided similarly to the previous group. An example of the orcinol type is physodic (VIII) and of the β-orcinol type stictic acid (IX). Fumarprotocetraric acid, with related acids, belongs to the β-orcinol derivatives. At least 11 depsidones are known.

9. Anthraquinone derivatives

C. Those of unknown structure.



VIII



IX

The third group of the systematics — sugar alcohols — actually cannot be classified among the lichen substances in their original sense as they are not encountered in lichens alone. The precise formulae of the compounds in Groups 2, 6, and 9 (Zeorin, Thiophene and Anthraquinone Groups) are not known to date.

In spite of the differences in structural formulae, lichen substances have a considerable number of properties in common. As a rule, they only contain carbon, hydrogen and oxygen; the only definite exceptions are the chlorine-containing chloratranorin [Koller and Pöpl (45), Pfau (62)] and diploicin and gangaleoidin [Nolan *et al.* (54)]. All lichen substances proper are crystallising, in most cases acid in character (which accounts for the widely used denomination lichen acids), and even in the form of alkaline salts their solubility in water is very poor; *e. g.* usnic acid is soluble in water at 19° C 1:5 mill., as sodium salt 1:600 (27). Several lichen substances are optically active — this applies to all the aliphatics (71); usnic acid even has an unusually high specific rotation. The colour of the crystals varies from colourless to white and reddish yellow. Several lichen substances have a very bitter taste.

The amount of lichen substances contained by the different species varies greatly. From Parmelia coralloidea 23.5 per cent of lecanoric acid alone was obtained, from Lepraria chlorina 10.5 per cent of vulpic acid, from Alectoria ochroleuca 5.5 per cent of usnic acid (96), whereas *e. g.* the contents of the protolichesteric acids in Iceland moss amounts to a few promilles only. The normal amount is possibly around 1 per cent.

On the Medicinal Use of Lichens

A. POPULAR USES

In popular medicine the use of lichens is of ancient origin. Evernia furfuracea was apparently used for medicinal purposes in Egypt in the 18th and 17th centuries B. C., and is still brought to Egypt, in the present century, together with Iceland moss, as a foreign drug from Europe (59). Hippocrates recommended Usnea barbata for uterine trouble (47); the same lichen (Usnea longissima) was employed by the Chinese, under the name of »Sun-Lo», as an expectorant, and its surface powder for the treatment of ulcers (82). The Manchurian 'Shi-hoa' medicine contained *e. g.* obtusatic acid of depside type (59). The Malayans still employ the Usnea species medicinally for colds and as a tonic (59).

In the 15th century A. D. lichens constituted an important commercial article in Europe. In the 18th century Peltigera canina was sold as »pulvus antilyssus», and the famous medicine »Lichen quercinus virides» contained mainly Evernia prunastri, E. furfuracea and Parmelia physodes. Lichen islandicus, at that time, was a remedy held in high esteem, and »Mucus cranii humani», lichen grown on the human skull, »cost its weight in gold» (59). Iceland moss and Lobaria pulmonaria were generally used for the treatment of catarrhal hemoptysis and pulmonary tuberculosis, *e. g.* in the form of cough-tea or »lichen chocolate» (25, 32), the former also being employed as a laxative (23). Xanthoria parietina, Cladonia and Pertusaria species also »helped» in the most varied range of diseases: fever (34), jaundice (48), epileptics, convulsions (59), »gout and other diseases», and oedema (25) etc.

In Finland the popular medicinal use of lichens seems to have been very widely spread. In the collections of Finnish folk-lore lichens are very often mentioned in connection with different cures. A considerable part of such data, naturally, is of a purely magic

character, possessing no medical basis. Also, the definition of the different lichen species and of the complaints and diseases treated with lichen is for a great part very vague. But the folk-lore collected from different parts of Finland (98) shows a surprising compatibility, and in their main features the descriptions conform very closely with information obtained from other countries.*

As a typical example of an obviously non-medical indication of use may be mentioned the use of yellow lichen, on the basis of colour analogy, for the treatment of jaundice. Reports of such treatment exist from a number of localities (Vihanti [1, 2], Paimio [3], Artjärvi [4], Oinasjärvi [5], Pyhäjärvi [6], Mouhijärvi [7], Ahlainen [8], Suistamo [9], Alatornio [10]). The lichens have usually been taken from »the wall of an old barn», or from the surface of stones; the species are likely to be Xanthoria parietina and polycarpa, possibly also Cetraria pinastri. The lichens have either been eaten in their natural state or, more often, boiled in water or milk. Sometimes spirits is mentioned as the dissolving agent (Kangasniemi [11]). The »similia similibus» character of such medicinal use is evidenced by the general recommendation of yellow silk, thread, paper or anything yellow for the treatment of jaundice (Sotkamo, Kuhmo, Paltamo [12]).

Also probably based on the exterior appearance of lichen is the use of lung moss (Lobaria pulmonaria, popularly called »raijan keuhko, raijan rämmäleet, ketun keuhkot») mainly for the treatment of pulmonary tuberculosis and coughs (Hollola [13], Pyhäntä [14], Mouhijärvi [15], Vihanti [16], Karstula [17]. Ilomantsi [18], Vihti [19], Tammela [20]). The medicine is usually taken in the form of tea made of dried lichen, of which »the taste is terribly bitter» (Koskenpää [21]), which is apparently due to its content of stictic acid. The dose mentioned is a tumblerful (Kangasala [22]). The same

* The information quoted here is primarily taken from the collections of the Folklore Archives of the Finnish Literature Society on popular medicine, so far not arranged; it has been possible to peruse but a fraction of the material available. Source indications refer to the collections by each collector. A minor portion of the indications refers to the questionnaire 1948:26:1051 distributed by the periodical »Sanastaja», of the Finnish Dictionary Foundation (the relevant notes are prefixed with the abbreviation S, followed by the name of the supplier of the information). In addition, there are some personal reports (P).

[1]) Rautell 656; [2]) S: Mustakangas; [3]) Kallio 2164; [4]) Anttila 282; [5]) Krohn 14478; [6]) Nikki l 247; [7]) Saarelma b 8; [8]) S: Lindroos; [9]) Valve b 957; [10]) Kallio 3227; [11]) Kuitunen 915; [12]) Mustakallio 8; [13]) S: Saksala; [14]) S: Partanen; [15]) Saarelma 11; [16]) S: Mustakangas; [17]) S: Rautiainen; [18]) S: Varis; [19]) S: Halen; [20] P: Elvi Pakarinen, 1928; [21]) S:

medicine is also recommended for various stomach ailments (Mouhijärvi [23], Pertteli [24]).

The easily recognisable Peltigera aphtosa (popularly termed »sampparuoho, sammaslehti, -ruoho, sampaheinä» etc.) is mentioned as a remedy for infantile aphtae, also obviously for reasons of exterior resemblance (Pyhämaa [25], Tammela [26], Rauma [27], Humppila [28]), and for coughs (Somero [29]). In Norway the same species is used in the treatment of eruptions (48).

Lichens with the widest range of medical uses in Finland are probably the beard mosses (actually the genera Usnea and Alectoria, perhaps also Ramalina thrausta), maybe in part due to the general spread of their growth. The advice given is to place a bunch of lichen on the fresh wound, to »heal and draw off the pain» (Humppila [30], Kaavi [31]), or to bathe it in water boiled with lichen (Kaavi [32], Ylikainuu [33]). The same methods of treatment are recommended for infected wounds or purulent inflammations (popularly termed as »ruusu» (erysipelas?) (Pieksämäki [34], Lapua and Kuortane [35], Kivijärvi [36]), and particularly for athlete's foot (Jämsä [37], Viipuri rural commune [38], Oulu [39]). For various eruptions, particularly if they are »caused by the wind» (popularly termed »tuulen lento, tuulimato, tuulimaahinen») beard mosses are also believed to help (Korpilahti [40], Pielisjärvi [41], Virrat [42], Kiisjoki [43], Nousiainen [44], Pihtipudas [45], Lapua [46]), as well as for »savipuoli» (pityriasis?) (Huittinen [47]). Users are also advised to place beard moss in water in which a sick child is to be washed, to »remove the curse» (Ruovesi [48, 49], Vienankarjala [50], Haapamäki [51]). According to occasional scraps of information, beard moss decoctions have been used to treat whooping cough (Kangasniemi [52]), throat ailments (Haukivuori [53]), ague (Längelmäki [54]), jaundice (Kangasniemi [55]), dysenteric diarrhoea (Suistamo [56]), tooth ache (Lehtimäki [57]) and various other complaints (Pihtipudas [58], Etelä-Pohjan-

Vuojansalo; [22]) S: Franssila; [23]) Saarelma 11; [24]) Kallio 2796; [25], [26], [27]) P: E. Pakarinen, 1928; [28]) S: Sillanpää; [29]) P: E. Pakarinen, 1928; [30]) S: Sillanpää; [31]) Teräsvuori 659; [32]) same 656; [33]) Meriläinen II o 1650; [34]) Lång 469; [35]) Rosnell k 50; [36]) Krohn l 3567 a); [37]) Nieminen b 234; [38]) S: Vakkinainen; [39]) Paulaharju 4851, 4853; [40]) Kotikoski 92; [41]) S: Kontkanen; [42]) Tarkkila 669; [43]) Meriläinen II 178; [44]) S: Leivo; [45]) Krohn 16500, e 4078; [46]) Tervo 315; [47]) Saarelainen 38; [48]) Saariluoma 3380, 3381; [49]) Saariluoma b 3406; [50]) Paulaharju p 5957; [51]) Salokannel 223; [52]) Kuitunen f 882; [53])Vidbom f 165; [54]) Kivi 128;[55]) Kuitunen f 917; [56]) Valve i 288; [57]) Vallinmäki k 146; [58]) Pihtipudas K.S. 587, 588; [59]) Et.

maa [59], Savo-Karjala [60]). To staunch hemorrhages, lichen dipped in blood has been burned and the ashes placed in the wound (Niskajoki [61]). Advice for the treatment of a sty is to remain in the smoke rising from smouldering lichen (Konginkangas [62]).

The heath lichens growing on the ground and on rocks have also been put to several uses: Iceland moss (Cetraria islandica) (Nousiainen [63]) and reindeer moss (Cladonia alpestris, sylvatica and rangiferina) are mentioned as remedies for pulmonary tuberculosis and coughs (Maaninka [64], Pyhäranta [65], Nakkila [66], Nousiainen [67], Laihia [68]); the remedy was taken either boiled or in the form of so-called lichen milk. These lichens are also known to have been used for various inflammations (Inkeri [69], Haapavesi [70], Korpiselkä [71]) and in washing water for babies (Suojärvi [72]).

The information received on so-called cup-lichens (Cladonia coccifera, deformis etc.) is on similar lines: for cough remedies (Pyhäranta [73], Kuusamo [74]), chest pains (Niemenkylä [75], Rautavaara [76], Tuusniemi [77], Pudasjärvi [78], Perniö [79], Russian Karelia [80], [81], Viitasaari [82], Kainulaisjärvi [83]), throat pains (Siikajärvi, Nilsiä [84]) and for infantile aphtae (Jokioinen [85], Ruovesi [86], Muurla [87]).

Less frequently than the above, stone mosses (Parmelia, Stereocaulon, etc. species) are recommended as remedies for wounds (Alvejärvi [88]), coughs (Inkeri, Tyrö [89]) and eruptions (Viljakkala [90], Utajärvi [91]).

In cattle breeding also lichens have a great number of popular uses. Internally, they are believed to help a cow in various diseases (»pistokseen, ähkyyn, ammuksiin», Russian Karelia [92], [94], Nilsiä [93]; »lentoa varten», Lohja [95]; »lähätystautiin», Russian Karelia [96]), in keeping the cows at home and propitiating them to other domestic animals (Saarijärvi [97], Russian Karelia [98], Kittilä [99]), and to give them an appetite (Joutsa [100]). This last-mentioned use, considering

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Pohjanmaan N.S. k 122; [60]) Pääkkönen f 290; [61]) Meriläinen l 1742; [62]) Leskinen e 8; [63]) S: Leivo; [64]) Rytkönen 697; [65]) Koskinen 397; [66]) S: Grönroos; [67]) S: Nousiainen; [68]) S: Koskinen; [69]) Pääkkönen 98 a; [70]) Keski-Pohjanmaan kans.op.: Murto; [71]) Mikkonen 241; [72]) Krohn 6171 d; [73]) Koskinen a 395; [74]) Marttinen 1618; [75]) Krohn 16571; [76]) Krohn 1380; [77]) Krohn 13829 c; [78]) Rautell 850; [79]) Myrsky a 331; [80]) Meriläinen 183; [81]) Krohn 3567; [82]) Moisio 283; [83]) Meriläinen II o 1511; [84]) Krohn 10519 b; [85]) Vihervaara d 1100; [86]) Saariluoma b 3386; [87]) Saariluoma a 1668; [88]) Krohn 15549; [89]) Putkonen 119; [90]) Lahdenmaa 22; [91]) Marttinen 1605; [92]) Meriläinen 1042; [93]) Krohn 14558; [94]) Krohn 13515; [95]) Österberg M 54; [96]) Krohn 16184; [97]) Karhumäki 87; [98]) Krohn 13778; [99]) Paula-

the bitter taste peculiar to lichens, may have its physiological basis. Understandable also is the effect of lichens in inflammation of the udders (Inkeri [101], Liperi, Eno, Ilomantsi [102]), in wounds (Kainulaisjärvi [103]) and in sores (Kärsämäki [104]). More difficult to explain is the magical belief according to which a milch cow can be made dry by feeding it with the beard moss of dry trees, and restored by feeding it with the beard moss of fresh alder (Muujärvi [105]). A similar antagonism is suggested by the following: if curdled milk is burned and then placed in the churn, the churn will produce no butter. The spell is counteracted by washing the churn with a decoction of lichen taken from a »weeping rock» (Ylikiiminki [106]).

As can be seen from the above, in the popular medicinal use of lichens superstition and a primitive medical skill, based on nature and perhaps on rough empiricism, are always mixed up. It is of interest to note that, on the use of lichens for coughs or expressly for pulmonary tuberculosis, old folk-lore exists from different parts of the globe, and that present-day science too has found fairly strong antibiotics against the TB bacillus in the same species to some extent (Usnea species, Cetraria islandica). As to the lichen species mentioned above, which have obviously been popular as medicines, no lichen acids proper have been found in Peltigera aphtosa, and the lichen substances contained in Lobaria pulmonaria and in the Xanthoria species have not been found to be active; the attention devoted to these species, however, is readily explicable from the general rule »similia similibus». But Cladonia alpestris, silvatica, coccifera and deformis, Ramalina thrausta, Evernia prunastri and all Usnea species contain active usnic acid, Cetraria islandica d-protolichesteric acid, Parmelia physodes and Evernia furfuracea physodic acid and Cetraria pinastri vulpic and pinastric acid (96), all of which have been found active. The concentration of the active substances, it is true, is not very high, but the effect is enhanced by the fact that the lichen substances primarily exist on the exterior surface of the thallus in the form of powder or crystals [the commonest active substance, usnic acid, always (89)], and hence, although not readily soluble, they can diffuse into their surroundings.

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harju 15429; [100]) Lilius 222; [101]) Lukkarinen 871; [102]) Manninen j 682; [103]) Meriläinen II o 1502; [104]) Keränen l 128; [105]) Meriläinen II 748; [106]) Meriläinen 193.

B. PHARMACOLOGICAL STUDIES OF LICHENS

The lichen medicines in old pharmacopoeias are compatible in their character with popular traditions. The oldest European pharmacopoeia (of 1546), includes no lichens among the drugs listed (84), whereas the »Pharmacopoea universalis» of 1846 lists a great number of lichen medicines. The following species are mentioned: Cetraria islandica and nivale, Cladonia coccifera and pyxidata, Usnea plicata, Peltigera canina, venosa, horisontalis and polydactyla, Lobaria pulmonaria, Xanthoria parietina and Evernia prunastri. According to this work, Iceland moss has been included among the drugs listed in 50 contemporary pharmacopoeias or dispensatories, recommended in the first place as a cough cure (70). Lichens have gradually disappeared from the list of official medicines; *e.g.* the »Pharmacopoea Fennica» excluded Lichen islandicus in 1914 (66) and the corresponding Danish pharmacopoeia in 1948(63). However, at least the Japanese pharmacopoeia of 1922 (69), the Estonian of 1937 (65) and the French, Swiss and German pharmacopoeias valid at present (67, 68, 64) include Iceland moss as a drug, the French one both as a paste and a potion.

Several lichen acids have been studied from the pharmacological point of view. Alms, as early as 1832, recommended picrolichenic acid, subsequently identified by Zopf, for the treatment of »Wechselfieber», prompted in the first place by its taste, reminiscent of quinine (1), and similarly Lebail later on (47).

Ramm (75) and Neuberg (53) carried out animal tests with cetrarin, finding it relatively harmless for mammals (lethal dose 0.2 g per kg of body weight). Injected in the form of sodium salt, the acid induced a specific acceleration in peristalsis, increased blood pressure and secretion of bile. According to Neuberg, accelerated peristalsis was also produced in the isolated bowels of a dog, and he recommended the substance as a remedy for anaemia and lack of appetite. Later on, cetrarin has been established to be an ethyl-protocetraric acid (42). Guesdon reported good results with cetraric and protocetraric acids as antiemetics *e.g.* in pregnancy and tuberculosis (24).

The vulpic acid contained in Letharia vulpina, used as fox poison,

has been repeatedly studied toxicologically. Kobert (40) and Neuberg (53) gave the lethal dose for mammals as 20—30 mg per kilogram; the same figure applies to the closely related pinastric acid. The toxicity of vulpic acid was later found to be lower; Santesson (1939), obtained 78.8 mg per kilogram as the lethal dose for a cat, the most noticeable symptom of poisoning being acute dyspnoea (77). Brodersen and Kjaer (1946), reported the lethal dose for a mouse as 75.0 mg per kilogram (13).

The early report on usnic acid produced by the Japanese Chiba (17) (1898) is of great interest: He reported that tinctures of Usnea species had achieved good results in the treatment of lymphadenitis tuberculosa colli. Mikoshiba (1933) published his investigations into the pharmacology of usnic acid and its derivatives; according to his studies, usnic acid has a papaverine-like effect on the smooth muscles, but is less poisonous than papaverine; the lethal dose for a mouse is 7.0 mg/10 g subcutaneously and 0.25 mg/10 g intravenously applied (= 0.7 and 0.025 g per kg) (82).

Fischer and Toth (22) investigated certain effects of lichesteric acid. The hemolytic index obtained by them for defibrinated blood was 40 000; the index was found to drop to 5 000 if equal amounts of lichesteric acid and cholesterin were added. The lethal dose obtained for a frog was 200 γ/g, and for a mouse, intravenously, 100 γ/g (0.2 and 0.1 g/kg), the fish index was 25 000, normal drop number 186, and foam value 1:25 000. Of the greatest interest is the ability of the acid, as found by them, greatly to promote resorption: with a frog, the symptoms of strychnine poisoning were produced percutaneously by 1/2—1/2.5 of the ordinary amount with a small simultaneous administration of lichesteric acid. The adsorption-increasing dose for lichesteric acid, in γ per g of a frog, was 3 γ after 45 min, obtained from the curare test. They presume that a part of the medicinal effects of Iceland moss are based on this property of lichesteric acid.

Huzikawa found in 1939—1941 that the phenols and their derivatives, components of lichen depsides and depsidones, possess remarkably strong antiseptic qualities (33).

C. PREVIOUS STUDIES OF THE ANTIBIOTICS OCCURRING IN LICHENS

Modern research into lichens, *e.g.* the study of lichens and lichen substances from the antibiotic point of view, only started a few years ago, after the various fungal groups, from the genera Penicillium and Aspergillus onwards, had been preliminarily investigated. The first qualitative study of the antibiotic properties of lichens was published by Burkholder *et al.* in 1944—1945. They tested 100 American lichen species in relation to Staphylococcus aureus and B. subtilis, by the Oxford Cup method, extracting a certain weight amount of lichen by phosphorus buffer solution. 52 species (52 per cent) prevented the growth of either one or both of the bacteria studied. With a few exceptions, the lichens studied had no effect at all on Gram-negative bacteria. The usnic acid isolated from Cladonia mitis prevented the growth of B. subtilis, but not of the staphylococcus or the colon bacillus (14). Stoll *et al.* (1947) boiled lichen for a short while with 5 per cent alkaline glucose solution, and made corresponding plate tests with the extract obtained: out of 58 Swiss species, 38 proved to be active against staphylococcus (65.5 per cent); clearly the most general active substance was usnic acid (88). In the plate tests with pieces of lichen by the present author in 1949, 52 out of 82 Finnish lichen species investigated revealed properties preventing the growth of various bacteria (63.4 per cent) (90).

Quantitative studies of crystalline lichen substances were initiated by Barry in 1946; he found that the chlorine-containing lichen substance, diploicin, previously isolated from Buellia canescens (54), inhibited the growth of human TB and diphteria bacillus 1:100 000, and that of Mycobact. smegmatis 1:70 000 *in vitro*. He also pointed out that the only compound of diphenyl-ether type (such as the product of alkaline hydrolysis of diploicin) occurring in a normal organism is thyroxin, but could not establish any definite physiological analogy between it and diploicin (11).

Subsequent studies have primarily dealt with usnic acid, in the first place its inhibiting effect on the growth of TB bacillus. In 1947, Marshak (49) isolated from the lichen Ramalina reticulata a crystalline substance which he subsequently found to be usnic acid

(50), completely inhibiting the growth of different strains of human TB 1:20 000—1:50 000, and weakening their growth 1:200 000—1:2 000 000. The growth of bovine TB was completely inhibited in 1:20 000, in which titre two avian strains were only partly inhibited. The growth of staphylo-, strepto- and pneumococci was inhibited in 1:20 000. The effect was evidently bactericidal. Stoll *et al.* (1947), obtained the following completely inhibiting titres for the usnic acid produced as a by-product from Cetraria islandica: human TB 1:64 000—1:800 000, bovine 1:500 000, an avian strain 1:125 000, Staph. aureus and Streptoc. pyogenes 1:100 000; no effect on colon bacillus, S. typhus and dysentery bacillus (88). In a third work published the same year, Bustinza and Lopez reported complete inhibition by the usnic acid isolated from Usnea barbata, in the form of sodium salt, of human TB cultivated on glycerine broth in 1:500 000, an avian strain 1:100 000. With Dubos's culture medium a 5—10 times greater concentration was necessary. Bustinza and Lopez tried to combine streptomycine and usnic acid into a salt but the result did not crystallise; possibly it was a mixture of usnicates, of very poor solubility (15). Shibata *et al.*, in 1948, compared the activities of usnic acid and its derivatives. With the different optic forms of usnic acid and their sodium salts, the inhibiting titre was, with an avian TB strain 1:160 000, with staphylococcus 1:160 000—1:320 000. Acetylation of hydroxyl groups reduces the effectivity to from a half to a quarter of that of the existing one (82). Marshak, Schaefer and Rajagopalan (1949) studied the activity of usnic acid and 33 related compounds, as well as of vulpic acid: only monoacetyl usnic acid against human TB obtained the same titre as usnic acid: 1:100 000 (52). The author (1949), for usnic acid isolated from Cladonia alpestris, obtained the completely inhibiting titre of 1:60 000, and a growth-retarding effect in 1:160 000 (90). Klosa (1949) reported the inhibitory titre of 1:1 000 000 for usnic acid against human TB, streptococcus and staphylococcus (36).

In 1948 Siintola *et al.* published a preliminary study of the pharmacological properties and production of usnic acid (83). Heilala and Siintola, in 1949, studied the solubilities of usnic acid and its sodium salt: the former dissolves in water at less than 1:5 mill., the latter at approx. 1:600. — In studying the isolation

of usnic acid from Cladonia alpestris they obtained the best results by using a mixture of alcohol and NaOH. — From the faeces of a patient who had taken usnic acid they demonstrated 36—65 per cent of the quantity taken (27).

Of the effect of usnic acid *in vivo,* few reports exist so far. In his first work quoted (1947) Marshak reported that usnic acid retards the course of tuberculosis in guinea-pigs (49); in a more extensive series of tests carried out by him later (1950), he could observe no retardation of the course of the disease in guinea-pigs treated with usnic acid alone, nor in those treated with a small amount of streptomycine (2 mg/day); but a large dose of streptomycine (6 mg/day) and combined treatment also (2 mg/day of streptomycine and, in addition, usnic acid) produced considerable retardation. The effect of usnic acid is based on the inactivation of desoxyribonuclease (51). Pätiälä *et al.* (1948) found that l-usnic acid had retarded the course of the disease in guinea-pigs inoculated with tuberculosis. In preliminary tests on patients they found that a daily dose of 3 g caused indefinite pains in the liver; a daily dose of 1 g produced no symptoms of poisoning. The treatment seemed to have an alleviating effect on throat irritation and dyspeptic troubles (74). In their paper published subsequently (1950) Pätiälä *et al.* found in a test series with 36 guinea-pigs, that inoculation tuberculosis, when treated with l-usnic acid per os, was different both macroscopically as regards the number and size of tubercles especially in the spleen, and microscopically as regards the amount of Ca, connective tissue and necroses. In animals inoculated with an exsudative TB strain this effect was more apparent (73). In 1949 Pätiälä published the report of a case of Lupus vulgaris which had considerably improved after two months' treatment with 1 per cent usnic acid solution and ointment: the treatment seemed to have a particularly good effect on the lesions in mucous membranes (72).

Precise information on the effects of other lichen substances is very scarce. Burkholder *et al.*, in 1944—1945, reported atranorin and fumarprotocetraric acid as inactive (14); Stoll *et al.* (1947) indicated that the effect of vulpic, d-protolichesteric, lichesteric, dihydrolichesteric, physodic and diffractic acids was somewhat similar to that of the usnic acid (88). In 1948 Cavallito *et al.*

reported on a dilution series made with aliphatic lactones, including protolichesteric, lichesteric, and dihydrolichesteric acids: TB 1:600 000—1:1 000 000, Streptoc. pyog. 1:30 000—1:50 000, Staph. 1:100 000, Cl. Welchii 1:200 000—1:2 000 000. The results were in compliance with those obtained with other aliphatic lactones (16). Klosa reported the inhibitory titre of evernic acid against TB, streptococcus and staphylococcus at 1:1 000 000 (36). The author published, 1950, a report of in vitro tests made with crystalline lichen substances against 11 bacteria: d-protolichesteric, d-lichesteric, lichestrylic, l-usnic, divaricatic and physodic acids proved to be distinctly active towards Gram-positive bacteria; whereas evernic, fumarprotocetraric and salazinic acids were inactive (91) *.

* In their publications subsequently obtained by the author, Shibata and Miura tested 23 lichen substances towards avian TB and Staph. aureus: of them, the protolichesteric, l-lichesteric, l-dihydroprotolichesteric and divaricatic acids, spherophorin, anziaic, perlatorinic, olivetoric, sekikaic, ramalinolic, boninic and lobaic acids and didymic acid with its related compounds were distinctly active towards the staphylococcus or both the bacteria studied, the caperatic and rangiformic acids, zeorin, lecanoric acid, atranorin, thamnolic, salazinic, psoromic, and fumarprotocetraric acids, as well as pannarin and endocrocin, fairly inactive (82 b).

Plan of Work

In vitro studies have been previously published only on diploicin, usnic acid and protolichesteric acid and the derivatives of the two latter, although lichens have been found to contain many other active compounds also. The results of these studies, furthermore, are very difficult to compare with each other, due to the different bacterial strains, culture media, amounts of inoculum etc. This is distinctly seen *e.g.* in the differences of the titres in which the usnic acid, according to the various investigators, inhibits the growth of human TB in vitro.

With a view to the above, the object of the present work is

to investigate the extent to which certain Finnish lichen species possess antibiotic properties and whether these properties are explicable from the lichen substances contained in the species,

to isolate and identify a number of different types of lichen substances, to find out which of the types have an affect inhibiting the growth of micro-organisms in vitro and to compare the results with those obtained previously.

Preliminary Tests

Lichen Material

The aim of the preliminary tests has been to provide some kind of picture of the antibiotic effects of Finnish lichen flora. Indigenous lichen species probably total some 1 500; of this total, 149 forms, or about 10 per cent, will be dealt with. The genera are presented in the taxonomic order employed by Fink (20), with the species of each genus in alphabetic order. The lichens investigated belong to twenty of the 46 families recorded by Fink. The number of species was restricted in the first place by the rarity of several lichens and by their limited site (*e.g.*, the Lapland lichens), as well as by the fact that they can be difficult to detach from their substratum and hence unsuitable for the method employed (even many widely spread lichens growing on stones). It was not considered advisable to make use of existing collections of lichens of older date, since the fact that the lichens varied in age and were old might have adversely affected the results.

The lichens used in the tests were collected from various parts of Finland in 1948—1950. The majority of the material (116 lichens) was collected by the author. Their species determination, generally, was effected by Prof. Ernst Häyrén (104, against which there is no special entry in the table). Veli Räsänen, Sc.D., identified 12 species collected by the author [the third column of the table bears the entry (R)]; in addition, he placed at the author's disposal 22 species identified by him (entry R). Lars Fagerström, M.Sc., provided 11 species for the tests (entry F).

The lichens collected were allowed to dry at room temperature, and were kept in the usual way in paper bags. As a rule, the preliminary tests with lichens were carried out within 1/2—2 months of the date

of collection, except for some of the lichens not collected by the author himself, which had been in collections for up to two years. Considering the longevity of lichens — it is generally known that even dried in herbaria they keep alive for years, even up to five years (89) — the material employed in these tests, or at least the majority of it, can be considered to have been living. Apart from the date of collection and locality, the second column of the table gives the site of each species as it has been found that the content of lichen substances may vary on different substrata (96).

Methods

The tests were made in a Petri's dish, using the inhibitory zone method. The endeavour has been to make the method simple and applicable even to small lichen samples, and to provide as natural conditions as possible for the lichens in the tests. For this reason no solvents have been used that might lead to the decomposition of lichen substances; the tests have been made direct on round pieces of lichen 5 mm in diameter, taken with a sterile punch, and of an average weight of 5 mg. With very small samples it has been necessary to use even smaller pieces.

Each lichen species was tested on 8 bacteria: Sarcina lutea, Staphylococcus aureus, Streptococcus hemolyticus, Corynebacterium dipht. mitis, Bacillus subtilis, B. megatherium, Escherichia coli and Proteus vulgaris. (Details on the strains are given on p. 39). More than half the material was tested as regards Pseudomonas aeruginosa also.

The nutrient medium used was ordinary broth agar, a layer approx. 2 mm thick, on a Petri's dish, except for the streptococcus and diphtheria bacillus, which were cultivated on a so-called sandwich dish (bottom layer broth agar, surface layer blood agar, overall thickness approx. 3 mm). Small changes in the thickness of nutrient medium did not seem to have any considerable effect on the results. The pH of the nutrient media was ascertained to be 7.5. Judging by the indicator paper at least the lichens were found to cause no change in pH in their surroundings.

The cultivation of the plates was effected by »seeding» the surface of the nutrient medium with a 24-hour old broth culture of each

bacterium. After the surface had dried, the lichen pieces to be studied were placed in the nutrient medium with their top surface level with the surface of the nutrient medium and their cut exterior surfaces in contact with the nutrient medium. Plates grown in an incubator at 37° C were inspected after 1—2 days, depending on the growth rate of the bacteria. When plates were allowed to grow longer no changes were found to have taken place in the inhibitory zones.

Results

The results are given in Table I. The entry + in the table indicates an inhibitory zone of 1—7 mm from the edge of the lichen outward, ++ an inhibitory zone of over 7 mm, (+) = bacterial growth weakened around the piece of lichen but no distinct zone, — = no inhibitory effect on bacterial growth observed. (Around the pieces of several lichens the growth of some bacteria was distinctly intensified, a phenomenon that will not be considered here.)

Against the lichen species that proved definitely active the last column of the table gives the active lichen substance identified in subsequent detailed tests, or the apparent active lichen substance. The data on the lichen substances contained in the species are, unless some other source reference is quoted, based on Zopf's extensive investigations (96, 97).

TABLE I

Lichen species	Date and site of collection	Identified by (see p. 23)
Dermatocarpon miniatum	6. 8.—48 Espoo, rock	
Calicium viride	19. 7.—49 Pyhäjärvi, pine	R
Coniocybe furfuracea f. vulgaris	14. 6.—49 Jämsä	R
Cyphelium viridescens f. prominula	3. 5.—49 Pielavesi	R
Sphaerophorus fragilis	8. 7.—48 Degerö, stone	
Diploschistes scruposus	15. 7.—48 Helsinki, stone	
Leptogium cyanescens	30. 6.—49 Saarijärvi, birch	R
» saturninum	13. 6.—49 Jämsä, rowan	R
Pannaria brunnea	10. 8.—48 Laukaa, moss	R
Lobaria pulmonaria		
Nephroma parilis	4. 8.—48 Laukaa, stone	
» resupinatum	10. 8.—48 Laukaa, stone	
Peltigera aphthosa	1. 8.—48 Elimäki, stone	
» canina	10. 8.—48 Laukaa, stone	F
» erumpens	20. 3.—48 Pukinmäki, rock	
» limbata	6. 8.—48 Espoo, stone	
» malacea	6. 12.—48 Espoo, moss	
» polydactyla	21. 7.—48 Elimäki, rock	
» rufescens	6. 8.—48 Espoo, stone	F
» scaprosa	12. 6.—47 Vehkalahti, rock	F
» variolosa	17. 8.—49 Ruotsinpyhtää, moss	(R)
Lecidea fuscoatra	15. 11.—48 Helsinki, stone	
Biatora granulosa	15. 11.—48 Kilo, humus	R
» Koskinenii	6. 7.—49 Kivijärvi, pine	
» symmictera	4. 8.—48 Laukaa, alder	R
Psora demissa	23. 8.—48 Kilpisjärvi, 700 m.	R
Cladonia alpestris	10. 12.—48 Degerö, rock	F
» alpicola	28. 8.—49 Vehkajärvi, rock	
» amaurocrea	18. 7.—48 Elimäki, stone	
» bacilliformis	3. 6.—48 Elimäki, rotted stump	
» botrytes	30. 6.—48 Elimäki, rotted stump	
» cariosa	10. 8.—48 Laukaa, stone	
» carneola	18. 7.—48 Elimäki, stone	R
» cenotea	1. 6.—48 Alavus, rotted stump	
» coccifera	11. 7.—48 Elimäki, rock	

PRELIMINARY TESTS WITH PIECES OF LICHENS, FOR KEY, SEE PP. 24, 25

Identified by (see p. 23)	Sarcina lutea	Staphyloc. aureus	Streptoc. β-hemol.	E. coli	Proteus vulgaris	Coryneb. dipht.mitis	B. subtilis	B. megatherium	Probable effective substance (in brackets, the lichen substances present in the active species, of weak or unstudied activity)
	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	−	−	
R	+?	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	+	+	*
	++	+	+	−	+	−	+	+	* Spherophorin
	+?	−	+	−	(+)	−	+	+	* Diploschistesic acid
R	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
R	−	−	−	−	+	−	−	−	(Stictic acid)
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	+?	−	−	−	+?	
	−	−	−	+?	−	−	−	+?	
F	−	−	−	+?	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
F	−	−	−	−	−	−	−	−	
F	−	(+)	−	−	−	−	−	+?	
(R)	−	−	−	−	−	−	−	−	
	+	+	(+)	+	+	+	(+)	+	* (Gyrophoric acid)
R	(+)	−	−	−	−	−	−	−	
	+	+	+	−	−	+	+	(+)	*
R	+	−	−	−	−	−	−	−	
	+	−	+	−	−	−	+	+	* Usnic acid
F	(+)	(+)	+	−	−	+	+	+	*
	++	+	+	−	+	+	++	+	* Usnic acid
	−	−	−	−	−	+	++	++	*
	+	+	+	+?	+	+	+	+	*
	−	−	−	−	−	−	−	−	
	++	+	+	−	−	+	++	+	*
R	+	+?	+	−	−	−	(+)	−	*
	++	+	+	−	−	+	++	+	* Usnic acid

TABLE I

Lichen species	Date and site of collection	Identified by (see p. 23)
Cladonia cornuta	6. 12.—48 Espoo, rock	
» deformis	18. 7.—48 Elimäki, rock	
» digitata	10. 12.—48 Degerö, rotted stump	
» fimbriata f. simplex	6. 12.—48 Espoo, roadside ditch	
» Floerkeana	15. 11.—48 Kilo, rock	
» furcata	15. 11.—48 Kilo, wood	
» » var. palamea	6. 12.—48 Espoo, rock	
» gracilis	18. 7.—48 Elimäki, stone	
» » var. chordalis	22. 7.—49 Ruotsinpyhtää, rock	F
» » var. dilatata	22. 7.—49 Ruotsinpyhtää, rock	F
» pleurota	15. 11.—48 Kilo, rock	
» rangiferina	4. 7.—48 Elimäki, rock	
» silvatica	29. 6.—48 Tyrväntö, shingle roof	
» squamosa	18. 7.—48 Elimäki, rock	
» turgida	22. 7.—48 Elimäki, rock	
» uncialis	10. 8.—48 Laukaa, rock	
Stereocaulon denudatum	8. 12.—48 Degerö, stone	(R)
» » var. digitata		R
» paschale	8. 8.—48 Laukaa, stone	
» subcoralloides Nyl.	8. 8.—48 Degerö, stone	(R)
Gyrophora deusta	15. 11.—48 Kilo, rock	
» hirsuta	14. 6.—49 Kuhmoinen, stone wall	R
» hyperborea	1. 8.—48 Elimäki, stone wall	
» polyphylla	1. 8.—48 Elimäki, stone wall	
» polyrrhiza	15. 12.—48 Kilo, rock	
» vellea	31. 10.—48 Pielisjärvi, Koli Slope	R
Umbilicaria pustulata	6. 12.—48 Espoo, rock	
Pertusaria amara	10. 12.—48 Degerö, birch	
» discoidea	6. 12.—48 Espoo, rock	(R
Parmularia muralis (Squamaria saxicola)	15. 7.—49 Ruotsinpyhtää, coast rock	F
Lecanora badia	15. 12.—48 Helsinki, stone	(R
» carpinea	29. 7.—48 Tyrväntö, arbor vitae	
» cenisea	21. 7.—48 Elimäki, stone wall	
» subfusca *allophana	22. 7.—48 Elimäki, aspen	
» varia	15. 8.—48 Elimäki, brush fence	
Ochrolechia alboflavescens	4. 8.—48 Laukaa, alder	(R
» androgyna	1. 7.—48 Elimäki, birch	(R
» parella var. tumidula	20. 8.—48 Saarijärvi	R

(Continued)

Identified by (see p. 23)	Sarcina	Staphyloc.	Streptoc.	Coli	Proteus	Dipht.	Subt.	Megath.	Probable effective substance
	−	−	−	−	−	−	−	−	
	++	+	+	−	−	+	++	++	* Usnic acid
	+	+	+	−	−	+	+	(+)	* (Thamnolic acid)
	−	−	−	−	−	+	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	+	
F	−	−	−	−	−	−	−	−	
F	−	−	−	−	−	−	−	−	
	++	+	+	−	−	+	+	(+)	* Usnic acid
	−	−	−	−	−	−	−	−	
	++	+	−	−	−	+	++	+	* Usnic acid
	−	−	−	−	−	−	−	+	
	−	−	−	−	+?	−	−	−	
	++	+	+	−	−	−	+	++	* Usnic acid
(R)	+	+	+	−	(+)	−	+	+	* (Atranorin, stictic acid)
R	−	−	−	−	−	(+)	−	+?	
	+	+	−	−	+	−	+	+	* (Atranorin)
(R)	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
R	−	−	−	−	+?	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	+?	−	−	−	−	
	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	+	+	* (Gyrophoric acid)
	++	+	+	−	−	−	+	+	*
(R)	+	+	+	−	(+)	+	+	+	*
F	+	−	−	−	−	+	−	−	* Usnic acid
(R)	+	−	(+)	−	−	−	−	+	*
	−	−	+?	−	−	−	−	−	
	+	−	−	−	−	−	−	+	* (Roccellic acid, atranorin)
	−	−	−	−	−	−	−	−	
	+	−	−	−	−	+	++	+	* Usnic acid
(R)	+	(+)	+	−	−	+	+	+	*
(R)	(+)	−	+	−	+	+	+	+	* (Gyrophoric acid)
R	+	(+)	+	−	−	−	+	+	*

TABLE I

Lichen species	Date and site of collection	Identified by (see p. 23)
Ochrolechia tartarea	7. 8.—48 Laukaa, rock	
Icmadophila aeruginosa	3. 5.—49 Pielavesi, pine stump	R
Haematomma ventosum	10. 12.—48 Degerö, stone	
Phlyctis agaelea	23. 7.—48 Elimäki, maple	
Parmeliopsis ambigua	15. 8.—48 Elimäki, brush fence	
» aleurites	15. 12.—48 Kilo, pine	
Parmelia centrifuga	4. 7.—48 Elimäki, rock	
» conspersa	1. 7.—48 Elimäki, stone wall	
» » var. corticola	1. 7.—48 Elimäki, stone wall	R
» exasperatula	22. 7.—48 Elimäki, spruce	
» fuliginosa	8. 8.—48 Laukaa, aspen	
» incurva	6. 12.—48 Espoo, rock	
» olivacea	20. 7.—48 Elimäki, birch	
» omphalodes var. panniformis	11. 4.—48 Herttuaniemi, rock	F
» » f. grisea		R
» panniformis	10. 12.—48 Degerö, stump	
» physodes	22. 77.—48 Elimäki, birch	
» saxatilis	18. 7.—48 Elimäki, stone wall	
» sorediata	8.—48 Laukaa, stone	
» stenophylla	15. 8.—48 Elimäki, stone	
» stygia	15. 12.—48 Kilo, rock	
» sulcata	1. 7.—48 Elimäki, birch	
» subaurifera	4. 8.—48 Laukaa, alder	
» tubulosa	21. 7.—48 Elimäki, rowan	
Cetraria chlorophylla	16. 8.—48 Elimäki, brush fence	
» commixta	6. 12.—48 Espoo, rock	
» glauca	11. 7.—48 Elimäki, spruce	
» islandica	7. 7.—48 Lahti, wooded heath	
» pinastri	13. 8.—48 Elimäki, brush fence	
» tenuifolia	15. 8.—48 Elimäki, brush fence	
Cornicularia aculeata	15. 11.—48 Kilo, rock	
» odontella	16. 8.—48 Ruotsinpyhtää, rock	F
Evernia furfuracea	10. 8.—48 Laukaa, pine	
» prunastri	11. 7.—48 Elimäki, spruce	
Alectoria chalybeiformis	18. 7.—48 Elimäki, telegraph pole	
» implexa	4. 7.—48 Elimäki, spruce	
» » f. fucidula	5. 5.—49 Keitele	R
» jubata	22. 7.—48 Elimäki, birch	

(Continued)

Identified by (see p. 23)	Sarcina	Staphyloc.	Streptoc.	Coli	Proteus	Dipht.	Subt.	Megath.	Probable effective substance
	−	−	−	−	+	−	+	+	* (Gyrophoric acid)
R	+	−	−	−	−	−	+	+	*
	++	+	+	−	+	−	+	+	* Usnic acid, divaricatic acid
	−	−	−	−	−	−	−	−	
	++	+	+	+	+	−	+	+	*
	−	(+)	−	−	+	−	−	−	
	+	+	(+)	−	−	−	+	+	*
	++	−	−	−	−	−	+	+	* Usnic acid
R	+	−	−	−	+	+	+	+	* Usnic acid
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	++	+	(+)	−	−	−	+	+	* Usnic acid
	−	−	−	−	−	−	−	+	
F	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	−	−	
	++	+	+	−	+	+	+	(+)	*
	+	+	−	−	−	−	+	+	* Physodic acid, (atranorin)
	−	−	−	−	−	−	−	−	
	+	+	−	+	+	+?	+	−	*
	+	−	+	+?	−?	+	+	+	*
	−	−	−	−	−	−	−	−	
	+	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	+	+	−	−	+	+	−	+	* Physodic acid, (atranorin)
	−	+	+	−	+	−	+	+	* Protolichesteric acid, (atranorin)
	−	−	−	−	−	−	−	−	
	−	−	−	+	+	−	−	−	* (Atranorin)
	+	+	+	+	+	−	−	+	* Protolichesteric acid
	++	+	+	+?	+	+?	++	+	* Usnic acid, pinastric acid, vulpic acid
	−	−	−	+	+	−	−	−	*
	+	(+)	+?	−	−	−	−	+	* Protolichesteric acid
F	++	++	+	−	−	+	++	++	*
	+	+	+	−	−	−	+	+	* Physodic acid
	++	+	+	−	−	−	++	+	* Usnic acid, (evernic acid, atranorin)
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	+?	−	
	−	+?	−	−	+	−	−	−	

TABLE I

Lichen species	Date and site of collection	Identified by (see p. 23)
Ramalina farinacea	29. 6.—48 Tyrväntö, oak	
» fraxinea	22. 7.—48 Elimäki, aspen	
» nervosa	18. 8.—49 Ruotsinpyhtää, willow	F
» obtusata	29. 6.—48 Tyrväntö, oak	
» » f. baltica	4. 7.—49 Karstula, aspen	R
» pollinaria	6. 12.—48 Espoo, rock	
» polymorpha	18. 8.—49 Ruotsinpyhtää, rock	F
» populina	17. 1.—48 Herttuaniemi, maple	
Ramalina thrausta	20. 5.—48 Elimäki, spruce	
Usnea comosa	11. 7.—48 Elimäki, spruce	
» dasypoga	21. 7.—48 Elimäki, spruce	
» » var. spinosissima Räs.	11. 7.—48 Elimäki, rotted pine	(R)
» » var. tuberculata (Mot.) Räs.	4. 7.—48 Elimäki, pine	(R)
» » var. stramineola (Mot.)	9. 4.—50 Paavola, spruce	R
» fulvoreagens	18. 7.—48 Elimäki, birch	(R)
» glabrescens	18. 7.—48 Elimäki, spruce	
» hirta	2. 7.—48 Elimäki, birch	
» monstruosa	22. 7.—48 Elimäki, birch	(R)
» rugulosa	4. 7.—48 Elimäki, spruce	(R)
» similis	9. 4.—50 Paavola, spruce	R
Caloplaca aurantiaca	22. 7.—48 Elimäki, aspen	
Xanthoria parietina	3. 6.—48 Herttuaniemi	
» » f. chlorina	17. 5.—49 Jämsä, spruce	R
» polycarpa	29. 6.—48 Tyrväntö, birch	
Buellia disciformis	4. 8.—48 Laukaa, alder	
Physcia aipolia	16. 8.—48 Elimäki, aspen	
» ascendens	22. 7.—48 Elimäki, aspen	
» caesia	15. 8.—48 Elimäki, brush fence	
» obscura	4. 8.—48 Laukaa, aspen	
» pulverulenta	22. 7.—48 Elimäki, aspen	
» stellaris	15. 8.—48 Elimäki, rowan	
» » var. rosulata	17. 5.—49 Jämsä, spruce	R
» tenella	22. 7.—48 Elimäki, birch	
» tribacea	22. 7.—48 Elimäki, birch	
Anaptychia ciliaris	22. 7.—48 Elimäki, aspen	
Lepraria flava	1. 8.—48 Laukaa, under-surface of stone	
Crocynia membranacea	18. 7.—48 Elimäki, stone	
» neglecta	6. 12.—48 Espoo, rock	

(Continued)

Identified by (see p. 23)	Sarcina	Staphyloc.	Streptoc.	Coli	Proteus	Dipht.	Subt.	Megath.	Probable effective substance
	++	+	−	−	−	+	++	+	* Usnic acid
	++	−	−	−	−	−	++	+	* Usnic acid
F	+	(+)	−	−	−	−	++	+	* Usnic acid
	++	+	−	−	+	+?	++	+	* Usnic acid
R	+	+	+	−	−	+	+	+	* Usnic acid
	++	+	(+)	−	−	+	(+)	+	* Usnic acid
F	++	+	+	(+)	(+)	−	++	++	* Usnic acid
	+	+	+	−	−	−	+	+	* Usnic acid
	++	+	+	−	−	−	++	+	* Usnic acid
	++	+	+	−	−	−	+	+	* Usnic acid
	++	+	+	−	−	+	++	+	* Usnic acid
(R)	++	+	−	−	−	+	+	+	* Usnic acid
(R)	++	+	+	−	−	+?	++	++	* Usnic acid
R	+	+	+?	−	−	+	+	+	* Usnic acid
(R)	++	+	(+)	−	−	(+)	+	+	* Usnic acid
	++	+	+	−	−	−	++	+	* Usnic acid
	++	+	+	−	−	+	++	++	* Usnic acid
(R)	++	+	−?	−	−	+	+	(+)	* Usnic acid
(R)	++	+	+	−	−	+	+	+	* Usnic acid
R	+	+	+	−	−	+	+	+	* Usnic acid
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
R	−	−	−	−	−	−	−	−	
	+	−	−	−	−	−	−	+	
	−	−	+?	−	−	+?	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	+	+	(+)	−	++	−	+	+	* (Atranorin, zeorin)
	−	−	−	−	−	+	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	+	−	−	−	
R	−	−	−	−	−	−	−	−	
	+?	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	−	−	−	−	−	−	−	−	
	+	−	+	+	+	−	+	−	* Pinastric acid
	+	−	+	−	−	−	−	−	*
	−	−	−	−	−	−	−	−	

Discussion

Of 149 lichen species, 75 had a definite growth-inhibiting effect on a minimum of two bacteria studied (indicated in the table by *), the effect of 21 was weak or indefinite, and 53 had no effect at all. Considering the active substances contained in the species, the following summary can be obtained:

Of the lichens definitely inhibiting growth,					
protolichesteric acid	is	contained	in	3 + probably in a further 2 species:	(5)
pinastric acid	»	»	»	1	(1)
usnic acid	»	»	»	35 + probably in a further 6 species:	(41)
physodic acid	»	»	»	3	(3)
The active substance is known in a total of				42 + probably in a further 8 species:	(50)
The active substance is unknown in a total of				33	(25)

Dinstinctly positive results, total	75
Weak or indefinite	21
Negative	53
Total of lichens investigated	149

The 4 lichen substances mentioned have been found to be active in the later part of this investigation as well. It is remarkable that all species known to contain these lichen substances have proved distinctly active in these tests, in spite of the very poor solubility of these acids. Possibly the acids form salts in the weakly alkaline nutrient medium; in any case, the solubility of the substance seems to play no decisive part in tests carried out in this way.

It was ascertained that *e.g.* the genus Peltigera, in which so far at least no lichen substances proper have been found (96), and the genera Gyrophora and Umbilicaria, containing gyrophoric and umbilicaric acids which are very weakly active only (p. 53—54), in these tests too yielded negative or indefinite results only.

The effect of lichens containing usnic acid has been inhibitory to the growth of Gram-positive bacteria, particularly to Sarcina,

Subtilis and Megatherium, slightly less distinct with Staphylococcus, Streptococcus and the diphtheria bacillus. Those containing physodic acid have, in principle, a similar effect. Lichens with protolichesteric acid seem to have a weak effect on Gram-negative rods also. On Pseudomonas aeruginosa, on which over half the lichens were tested, none of the lichen species was found to have any effect.

In the group »Active substance unknown» no indication was found as to the substances contained in 17 lichens. However, it seems obvious that Cetraria tenuifolia, representing the narrow-leaved form of C. islandica, and Cornicularia odontella, belonging to the same sub-genus as C. aculeata, contain protolichesteric acid; similarly it is apparent that Parmelia stenophylla and centrifuga, as well as Parmeliopsis ambigua, contain usnic acid, as do other yellowish-green parmeliae (96). On the same grounds the active substance in Cladonia alpicola, botrytes and carneola should be the usnic acid. (These probabilities are included in the figures given in brackets in the summary.)

Of the 16 remaining lichens of the group »Active substance unknown» Zopf (96) has made an analysis, but the substances recorded by him have either not been studied subsequently or not been proved as active. In this group, however, the activity of spherophorin contained in Sphaerophorus fragilis and diploschistesic acid contained in Diploschistes scruposus seems very probable, these acids being of the same type as divaricatic acid (3), found to be active (p. 51) *.

The growth-inhibiting effect of several pieces of lichen on Proteus vulgaris is more difficult to explain as the direct result of lichen substances, In his earlier work (90), the author found that such lichens very often contain atranorin or some other lichen substance of depside type. However, none of the five lichen substances of depside type investigated later on in the present work inhibited the growth of proteus to any considerable extent. On the other hand, the crystalline decomposition result, atranol, obtained from the hydrolysis of atranorin, proved to be active against Gram-negative rods also (p. 56). At least a part of the activity against proteus and colon bacillus

* In the publication by Shibata and Miura, subsequently obtained by the author, spherophorin was reported to inhibit the growth of Staph. aureus in a dilution of 1:80 000 (82 b).

of the lichen pieces is likely to be due to a hydrolytic decomposition of depsides occurred for one reason or another. Also favouring this explanation is the fact that the inhibitory effect on Gram-negative rods is not as stable and limited to the species as the activity of lichens in general. In control tests with lichens containing known active substances, roughly identical results were regularly obtained at different times and with different specimens, whereas *e.g.* with Physcia caesia specimens, some inhibited proteus growth, others did not. Also connected with hydrolytic decomposition products perhaps is the finding of Burkholder *et al.* on Cetraria glauca, the ether extract of which proved to be inactive, the water extract again weakly active against B. subtilis (14).

On the basis of these approximative qualitative tests, a good two-thirds of the antibiotic properties of the lichens are explicable from the lichen substances that they are known to contain, according to our present-day knowledge. The activity of the following 23 species remains unexplained: Cyphelium viridescens, Biatora granulosa and symmictera, Cladonia bacilliformis, cenotea and digitata, Stereocaulon denudatum and paschale, Umbilicaria pustulata, Pertusaria amara and discoidea, Lecanora badia and cenisea, Ochrolechia alboflavescens, androgyna, parella and tartarea, Icmadophila aeruginosa, Parmelia panniformis and sorediata, Xanthoria polycarpa, Physcia caesia and Crocynia membranacea. Whether the effect of these species is based on known active compounds, related compounds, or possibly on their decomposition results, or whether active substances of a new type are in question, cannot be resolved on the basis of the present tests.

Tests with Crystalline Lichen Substances

A. PREPARATION AND IDENTIFICATION OF LICHEN SUBSTANCES

The isolation of crystalline lichen substances necessarily requires fairly large amounts of lichen. Hence, the selection of lichen substances for detailed study has been greatly dependent on the lichen species available for collection in large enough quantities. Several lichen substances are known to be entirely missing from the Finnish species, and the lichens containing some of the substances are so small or so rare that they could not be considered. On the one hand the endeavour has been to extract, as far as possible, species that have proved active in the preliminary tests, and on the other to have the different groups in Asahina's systematics (2) represented. The zeorin, thiophene and anthraquinone groups, of which the chemical formulae have not been finally established to date, and the group of sugar alcohols, which does not belong to the lichen substances proper, have remained unrepresented.

Dr. Vincent C. Barry, Department of Chemistry, University College, Dublin, has very kindly placed at the author's disposal a sufficient amount of diploicin, isolated in England from Buellia canescens (54) and found by him in 1946 to be active (11); diploicin is representative of the rare chlorine-containing lichen substances. The anilide of pulvid acid was synthesised at the Helsinki University Department of Chemistry, usnolic and decarbousnic acids made of usnic acid, and atranol, hydrolytically produced from atranorin at the University of Technology Department of Chemistry.

The lichen quantity required for extraction has been kept dry, either whole or ground. These lichen species too were identified

by Prof. E. Häyrén. In the extraction were used, initially, less completely purified, so-called technical solvents, in the crystallising pro analysi-products. As far as possible, the extraction was effected by Soxhlet's apparatus. — The preparation of each lichen substance will be described in detail in connection with the results of dilution series made with rapidly growing bacteria.

The following criteria were used in the identification of crystallised compounds:

1. the compounds were isolated from lichens in which the compound in question, according to literature, is to be found, *)
2. melting point,
3. molecular weight by titration with alkali
4. specific rotation,
5. crystal shape and colour,
6. solubility.

In addition, from salazinic acid, which has no melting point, its hexa-acetate was prepared, and from d-protolichesteric acid was made a serial synthesis d-protolichesteric acid → d-lichesteric acid → lichesterylic acid.

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* Aliphatic roccellic acid, which according to Zopf (96) should be present in Ochrolechia tartarea, was not found in the lichen in question.

B. TESTS WITH RAPIDLY GROWING COCCI AND ROD-SHAPED BACTERIA

Methods

For the dilution series, a 1 per cent aqueous solution was made of each compound; because of their poor solubility they were generally titrated into a sodium salt by adding an equivalent amount of NaOH. From some substances an emulsion was made without NaOH, to forestall the possible decomposing effect of the base. It has often been necessary to add alcohol as a solvent, but never more than 50 volume per cent. The highest concentration of compounds (basic solution 1:5 000) used in the dilution series was made by adding 1.0 cc of 1 per cent lichen substance solution to 49 cc of broth *). From this solution a dilution series, with broth in negative powers of 2, was made. The pH of the broth, like that of the basic solution was ascertained to be 7.5. (For certain lichen substances, $\leqq 7$, because of the possible decomposing action of the base.)

The tests were made with the following strains:

Sarcina lutea (Nyb.), ...	an old strain from collections
Staphylococcus aureus hemol. 1055,	a fresh strain from routine samples
Streptoc. β-hemol. (Lep.)	an old strain from collections
Pneumoc. typ. 2 (242).....	» » » » »
Coryneb. dipht. mit. 2065/414491 ...	» » » » »
B. subtilis (12 Reinikainen)	» » » » »
B. megatherium (Reinikainen)	» » » » »
Escherichia coli (647)	» » » » »
Salmonella paratyphi B 21905/46 ...	» » » » »
Proteus vulgaris 1045	» » » » »
11. Pseudomonas aeruginosa (20/9)	» » » » »

The inoculation was made from the broth culture of each bacterium, grown for 24 hours, diluted with physiological saline solution as follows: Sarcina, Streptoc. and Pneumoc. 1:500, Staph., Dipht., Megath. and Subtilis 1:5 000, Gram-negative rods 1:50 000. The inoculum was 0.1 cc per 1.0 cc of each tube of the dilution series. The series were checked after 24—36 hours.

* Accordingly the maximal concentration of alcohol was 1 per cent in test tubes. In the control series this concentration had no effect on the growth of the bacteria studied.

Results

The compounds isolated are reported in the order of Asahina's systematics. In this work attention has been paid to the bacteriostatic effect of the substances only. Where distinct differences have existed in growth intensity in the various tubes of the dilution series, this is indicated by + and ++. (Growth of controls = ++.) A smaller-size $^{+}$ and $^{-}$ sign in the subtilis column refers to the distinctly visible surface growth of the bacterium, which generally seems to be inhibited previous to complete inhibition of growth. (++) = variations in test results at different times.

Fatty Acids and Lactones

Caperatic acid, $C_{21}H_{38}O_7$

$$H_3C \cdot (CH_2)_{13} - \underset{\underset{COOH}{|}}{CH} - \underset{\underset{COOH}{|}}{C(OH)} - \underset{\underset{COOCH_3}{|}}{CH_2}$$

The caperatic acid is the monomethyl ester of tetradecylcitric acid.

The acid is isolated from Cetraria glauca (collected at Elimäki from spruce branches in 1948) mainly according to Zopf (96): the

TABLE II

THE DILUTION SERIES OF CAPERATIC ACID, FOR KEY, SEE P. 40

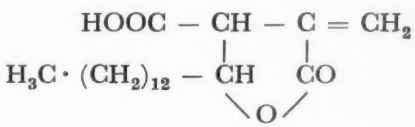
	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	++	++	++	++	++	++	++	++	++	++
Staph. ...	++	++	++	++	++	++	++	++	++	++	++
Streptoc.	−	−	++	++	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht. ...	−	−	++	++	++	++	++	++	++	++	++
Megath.	+	++	++	++	++	++	++	++	++	++	++
Subtilis ...	++$^{-}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$	++$^{+}$
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

lichen is extracted with ether (Soxhlet) and evaporated to dryness. The extract is then treated with boiling benzene in which atranorin is dissolved. The undissolved part is dissolved in cold absolute alcohol, heated and precipitated with water: thin, slightly yellowish leaves, m.p. 129.5—131°C (Zopf: 131—2°C).

Titration: 102.5 mg crystals (water, phenolphthalein); 4.75—4.80 ml 0.1-n NaOH were consumed. Molecular weight as dibasic acid 428 (calculated M = 402).

1 per cent aqueous solution, containing equivalent amount of NaOH, slightly yellowish, of which the basic solution 1:5 000, pH 7.5, was made. — Table II.

d-Protolichesteric acid, $C_{19}H_{32}O_4$



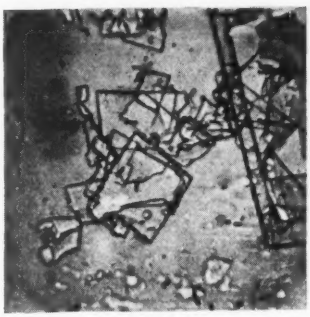


Fig. 1. Crystals of d-protolichesteric acid from glacial acetic acid, × 600.

d-Protolichesteric acid is a lactone carbonic acid with a long aliphatic carbon chain and double bond outside the lactone ring.

It was isolated from Cetraria islandica (collected from the Island of Degerö in the neighbourhood of Helsinki in 1949). Preparation in the main according to Zopf (96): the lichen was extracted with ether (Soxhlet), evaporated to dryness; the brownish green resinous substance obtained was washed with benzene and dissolved in cold ether, the fumarprotocetraric acid remaining undissolved. The precipitate remaining after the ether had been evaporated was crystallised from benzene and glacial acetic acid: colourless leaves, m.p. 105—6°C. (Asahina: 105—6°C.) Yield approx. 1 pro mille.

TABLE III

THE DILUTION SERIES OF d-PROTOLICHESTERIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	−	−	−	−	(+)	++	++	++	++
Staph.	−	−	−	−	−	(+)	++	++	++	++	++
Streptoc.	−	−	−	−	−	−	−	++	++	++	++
Pneumoc.	−	−	−	−	−	−	++	++	++	++	++
Dipht. ...	−	−	−	−	−	−	(++)	(++)	++	++	++
Megath.	−	−	−	−	−	−	(++)	++	++	++	++
Subtilis ...	−	−	−	−	(++)	++−	++−	++±	+++	+++	+++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat.	+	++	++	++	++	++	++	++	++	++	++
Proteus ...	+	++	++	++	++	++	++	++	++	++	++
Pseudom.	+	++	++	++	++	++	++	++	++	++	++

$[\alpha]_D^{20} = +11.9°$ (10.9 mg in 1 cc chloroform). (Asahina: +11.4 −12.8° at different temperatures.) — Fig. 1.

Titration: 54.8 mg crystals (asetone, phenolphthalein); 1.70 ml 0.1-n NaOH were consumed. Molecular weight as monobasic acid 322 (calculated M = 324).

1 per cent aqueous solution, containing equivalent amount of NaOH, colourless; basic solution made 1:5 000. — Table III.

If stored as 1 per cent sodium salt solution, d-protolichesteric acid seems to lose its activity considerably: in dilution series made from a solution kept for two months in a refrigerator, for instance, the minimum inhibitory titre obtained for staphylococcus was 1:5 000, and for subtilis 1:10 000. Slight weakening could often be noticed even in test series made on consecutive days. This phenomenon was not observed with the two following lichen substances, the isomer of d-protolichesteric acid, d-lichesteric acid, and lichesterylic acid.

d-Lichesteric acid, $C_{19}H_{32}O_4$

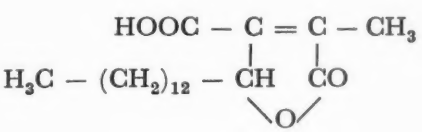


Fig.2. Crystals of d-lichesteric acid from glacial acetic acid, × 300.

The acid is an isomer of d-protolichesteric acid, with the double bond in the lactone ring. Preparation according to Asahina and Yasue (9): d-protolichesteric acid (m.p. 103—4°C) was heated for two hours, in an oil bath at 100—105°C, with acetanhydride. On cooling, cold water was added to the mixture, and it was filtered. The precipitate was crystallised from glacial acetic acid: colourless leaves, m.p. 123—4°C (Asano: 123.5°C). — Fig. 2.

Titration: 53.5 mg crystals (ethanol, phenolphthalein); 1.60 ml

TABLE IV

THE DILUTION SERIES OF d-LICHESTERIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	−	−	−	−	−	++	++	++	++
Staph. ...	−	−	−	−	−	++	++	++	++	++	++
Streptoc.	−	−	−	−	−	−	−	++	++	++	++
Pneumoc.	−	−	−	−	−	−	++	++	++	++	++
Dipht. ...	−	−	−	−	−	−	−	++	++	++	++
Megath.	−	−	−	−	−	−	++	++	++	++	++
Subtilis ...	−	−	−	−	−	(++)	++±	+++	+++	+++	+++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat.	+	++	++	++	++	++	++	++	++	++	++
Proteus ...	+	++	++	++	++	++	++	++	++	++	++
Pseudom.	+	++	++	++	++	++	++	++	++	++	++

of 0.1-n NaOH were consumed. Molecular weight as monobasic acid 334 (calculated M = 324).

1 per cent aqueous solution, containing equivalent amount of NaOH; basic solution 1:5 000, pH 7.5. — Table IV.

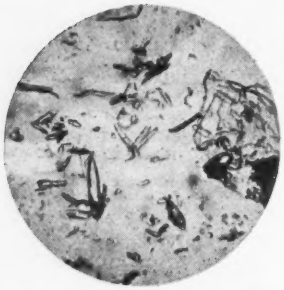
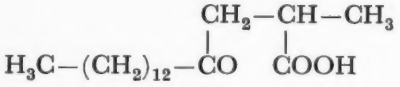


Fig.3. Crystals of lichesterylic acid from methanol, × 300.

Lichesterylic acid, $C_{18}H_{34}O_3$



In lichesterylic acid the lactone ring has opened; it is a long-chain α-methyl-γ-keto-monocarbonic acid.

Preparation according to Asano and Kanematsu (10): d-lichesteric acid (m.p. 123—4°C) was boiled for 4 hours, in a water bath, with

TABLE V

THE DILUTION SERIES OF LICHESTERYLIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	−	−	(+)	++	++	++	++	++	++
Staph. ...	−	−	−	++	++	++	++	++	++	++	++
Streptoc.	−	−	−	−	++	++	++	++	++	++	++
Pneumoc.	−	−	−	−	++	++	++	++	++	++	++
Dipht. ...	−	−	−	−	(+)	(++)	++	++	++	++	++
Megath.	−	−	−	−	−	++	++	++	++	++	++
Subtilis ...	−	−	−	−	−	++	++	++	++	++	++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

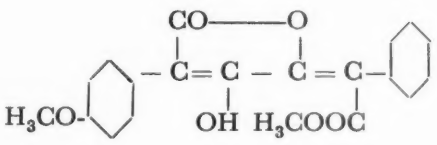
0.1-n KOH. It was precipitated with hydrochloric acid; the precipitate was washed with water and crystallised from methanol: white needles and narrow leaves, m.p. 79—81°C (Asano: 83—85°C). — Fig. 2.

Titration: 46.8 mg crystals (ethanol, phenolphthalein); 1.44 ml of 0.1-n NaOH were consumed. As monobasic acid the molecular weight is 298 (calculated M = 325).

1 per cent aqueous solution, containing equivalent amount of NaOH; basic solution 1:5 000, pH 7.5. — Table V.

Pulvic Acid Derivatives

Pinastric acid, $C_{20}H_{16}O_6$



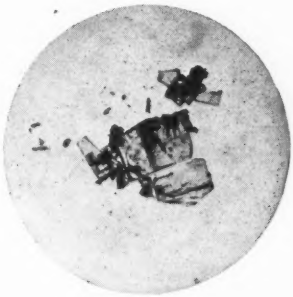


Fig. 4. Crystals of pinastric acid from ether, × 50.

TABLE VI

THE DILUTION SERIES OF PINASTRIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	–	–	–	(+)	++	++	++	++	++	++	++
Staph.	–	–	+	++	++	++	++	++	++	++	++
Streptoc.	–	–	+	++	++	++	++	++	++	++	++
Pneumoc.	–	++	++	++	++	++	++	++	++	++	++
Dipht.	–	(+)	+	++	++	++	++	++	++	++	++
Megath.	–	–	–	+	++	++	++	++	++	++	++
Subtilis ...	–	++⁻	++⁻	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺
Coli	(++)	++	++	++	++	++	++	++	++	++	++
Parat.	++	++	++	++	++	++	++	++	++	++	++
Proteus ···	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	+	++	++	++	++	++	++	++	++	++	++

Preparation from Cetraria pinastri (collected from stones and tree branches at Vaajakoski), according to Koller and Pfeiffer (43): the lichen was extracted with plenty of ether (Soxhlet) and filtered while warm. The filtrate was allowed partly to evaporate in the refrigerator, which resulted in the pinastric acid crystallising in beautiful orange-red crystals, while usnic acid and vulpic acid remained in solution. M.p. of crystals 198—200°C. (Koller and Pfeiffer, in vacuum: 203°C, Zopf 200—203°C, Hesse 196—198°C). — Fig. 4.

1 per cent aqueous solution, containing 50 per cent ethanol and

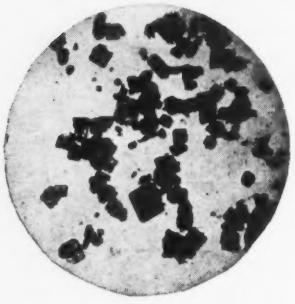


Fig. 5. Crystals of anilide of pulvic acid from toluene, × 50.

Anilide of Pulvic Acid, $C_{24}H_{17}O_4N$

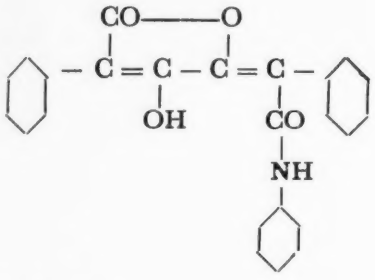


TABLE VII

THE DILUTION SERIES OF ANILIDE OF PULVIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	(+)	+	++	++	++	++	++	++	++
Staph. ...	−	−	(+)	+	++	++	++	++	++	++	++
Streptoc.	−	(+)	+	+	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht. ...	−	−	+	++	++	++	++	++	++	++	++
Megath.	−	−	−	−	+	++	++	++	++	++	++
Subtilis ...	−	−	−	(+)	+++	+++	+++	+++	+++	+++	+++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

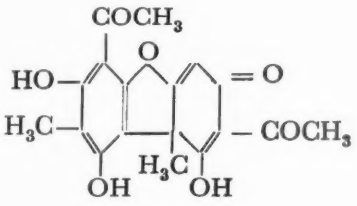
an equivalent amount of NaOH, bright orange-yellow in colour, basic solution made 1:5 000, pH 7.5. — Table VI.

Prepared at Helsinki University Institute of Chemistry as a part of serial synthesis from the anilide and the lactone of pulvic acid, according to Schenk (78): bright yellow crystals, m.p. 188—9°C. (Schenk 187≅8°C) — Fig. 5.

1 per cent solution, containing 50 per cent ethanol and 50 per cent 0.1-n NaOH, bright orange-yellow in colour. Basic solution 1:5 000, pH 7.5. — Table VII.

Cumarone Derivatives

l-Usnic acid, $C_{18}H_{16}O_7$



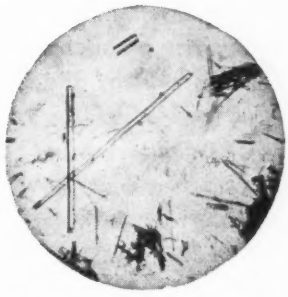


Fig. 6. Crystals of l-usnic acid from benzene, × 50.

TABLE VIII

THE DILUTION SERIES OF l-USNIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	−	−	−	−	(++)	++	++	++	++
Staph.	−	−	−	−	(++)	++	++	++	++	++	++
Streptoc.	−	−	−	−	−	++	++	++	++	++	++
Pneumoc.	−	−	−	−	(++)	++	++	++	++	++	++
Dipht.	−	−	−	−	−	(++)	++	++	++	++	++
Megath.	−	−	−	−	−	−	++	++	++	++	++
Subtilis ...	−	−	−	−	−	(++)	++	++	++	++	++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat.	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

Preparation from Cladonia uncialis (collected from the Island of Degerö in 1949), in the main according to Zopf (96); the lichen was extracted with asetone (Soxhlet), treated after evaporation with ether several times, thamnolic acid remaining undissolved. Crystallised from chloroform and benzene: light-yellow needle-like crystals, m.p. 197—8°C (Asahina: 202, Zopf 196°C). — Fig. 6.

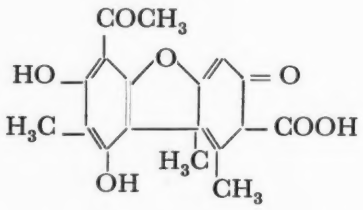
$[\alpha]_D^{20} = -492°$ (11.9 mg in 1 cc of chloroform). (Asahina, several results, +490—500° for d-usnic acid, Siintola *et al.* —452°C).

Titration: 69.6 mg of crystals (ethanol, phenolphthalein); 2.00 ml of 0.1—n NaOH were consumed. As a monobasic acid M = 348 (calculated M = 344).

1 per cent aqueous solution, with 40 per cent alcohol and an equivalent amount of NaOH; basic solution 1:5 000. The first two tubes of the dilution series were somewhat turbid owing to the poor solubility of the substance. — Table VIII.

Unanimity has not yet been reached regarding the structure of usnic acid. The above formula, considered as the most probable, has been given by Foster, Robertson and Healy (18).

Usnolic acid, $C_{18}H_{16}O_7$



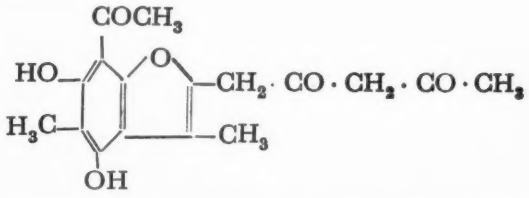
Prepared, like the above, at the Chemical Laboratory of the University of Technology according to Widman (94): l-usnic acid (m.p. 202°C) was kept at 59—61°C for 1 3/4 hours together with concentrated sulphuric acid. The solution obtained was poured, with vigorous stirring, into cold water, filtered and washed with water. The precipitate was boiled in alcohol for 4 hours, the filtrate evaporated, and the resinous substance obtained boiled twice with benzene to remove unchanged usnic acid. The final result was a farinaceous, yellow substance which distilled at 208—210°C as red steam.

Titration: 117 mg of crystals (water, phenolphthalein); 6.64 ml of 0.1-n NaOH were consumed. The molecular weight, as dibasic acid, 352 (calculated M = 344).

1 per cent aqueous solution, containing an equivalent amount of NaOH; basic solution 1:5 000, pH 7.5.

Usnolic acid was found to have no effect worth mentioning on the growth of the bacteria studied.

Decarbousnic acid, $C_{17}H_{18}O_6$



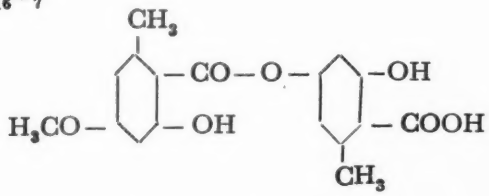
Prepared at the Chemical Laboratory of the Institute of Technology according to Widman (93): usnic acid, together with a small amount of alcohol, was heated for 6 hours at 150°C. The product was dissolved in ether, precipitated with alcohol and crystallised from alcohol: yellowish white crystals, m.p. 176°C (Shibata *et al.* 177°C) (48).

1 per cent aqueous solution, containing an equivalent amount of NaOH; basic solution 1:5 000, pH 7.5.

Decarbousnic acid produced no distinct growth-inhibiting effect on the bacteria studied.

Depsides of the Orcinol Group

Evernic acid, $C_{17}H_{16}O_7$



Prepared from Evernia prunastri (collected from birch trunks at Laukaa), mainly according to Hesse (30): the lichen was extracted for two days with ether (Soxhlet), the evaporated extraction treated

with chloroform and filtered. The precipitate was diluted in hot alcohol, when atranorin remained undissolved. On re-cooling of the alcohol the evernic acid precipitated, was crystallised again from acetone: small white crystals, m.p. 167°C (Hesse: 169°C).

Titration: 76.7 mg of crystals (ethanol, phenolphthalein); 2.25 ml of 0.1-n NaOH were consumed. As monobasic acid, the molecular weight is 341 (calculated M = 332).

1 per cent aqueous solution is prepared, with 20 per cent ethanol and an equivalent amount of NaOH. Like the corresponding solution of the following divaricatic acid, this will gradually turn pinkish in colour. In dilution series made as usual complete inhibitory effect with the dilution 1:5 000 against the bacteria studied is not visible, but the growth of Sarcina, Streptococcus, Megatherium, diphtheria bacillus and Subtilis, in dilutions of 1:5 000—1:10 000, is weaker than in the controls.

As it seemed probable that evernic acid is at least partly decomposed due to the effect of alkali (like divaricatic acid, see below), another series of tests was carried out, care being taken to ensure that the pH in no phase exceeded 7. A 1/2 per cent suspension was made from the acid by dissolving it in hot alcohol and dropping this

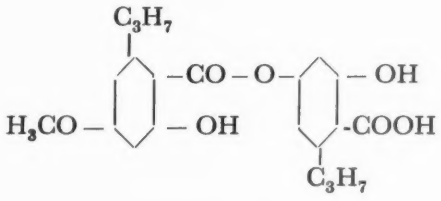
TABLE IX

THE DILUTION SERIES OF EVERNIC ACID, pH≦7, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	−	−	−	++	++	++	++	++	++	++	++
Staph. ...	−	++	++	++	++	++	++	++	++	++	++
Streptoc.	−	(+)	+	++	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht. ...	−	−	+	++	++	++	++	++	++	++	++
Megath.	−	(+)	+	++	++	++	++	++	++	++	++
Subtilis ...	−	(+)	++−	+++	+++	+++	+++	+++	+++	+++	+++
Coli	+	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	+	++	++	++	++	++	++	++	++	++	++

solution gradually into hot water. From the suspension the basic solution was made into broth, pH = 7. The solution 1:5 000 is initially slightly turbid, but clears up while the bacteria grow in the incubator. In dilution series carried out in this way, the evernic acid proves — admittedly weakly, but distinctly — active. — Table IX.

Divaricatic acid, $C_{21}H_{24}O_7$



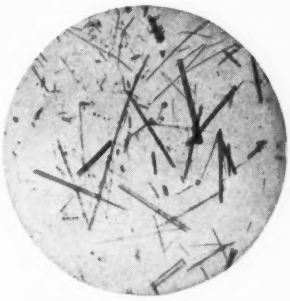


Fig. 7. Crystals of divaricatic acid from ethanol, × 50.

This acid differs from the former in its alkyl radicals only.

Prepared from Haematomma ventosum (collected from the Island of Degerö in 1949), according to Zopf and Asahina and Hirakata

TABLE X

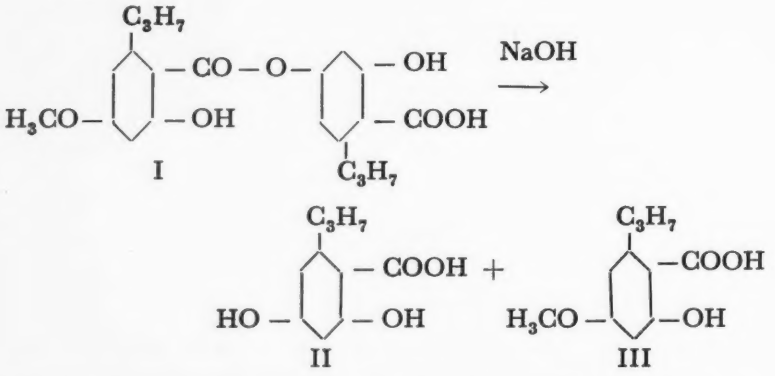
THE DILUTION SERIES OF DIVARICATIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	–	–	–	–	–	–	(++)	++	++	++	++
Staph. ...	–	–	–	–	(++)	++	++	++	++	++	++
Streptoc.	–	–	–	–	–	(++)	++	++	++	++	++
Pneumoc.	–	–	–	–	++	++	++	++	++	++	++
Dipht. ...	–	–	–	–	–	++	++	++	++	++	++
Megath.	–	–	–	–	–	–	++	++	++	++	++
Subtilis ...	–	–	–	–	–	(++)	+++	+++	+++	+++	+++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

(96,5): the lichen was extracted with cold ether, evaporated to dryness, crystallised from 60 per cent alcohol, acetic acid, benzene and acetone, the final crystallisation being from alcohol after treatment with animal charcoal: thin white needles, m.p. 136°C (Asahina 137°C). = Fig. 7.

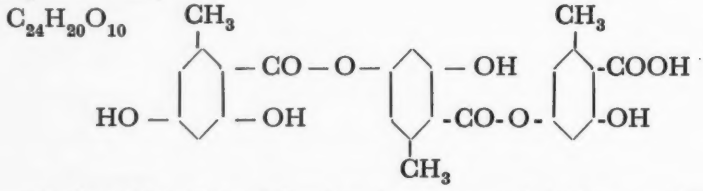
Titration: 53.7 mg of crystals (ethanol, phenolphthalein); 1.60 ml of 0.1-n NaOH were consumed. As monobasic acid, the molecular weight obtained is 336 (calculated M = 388).

1 per cent aqueous solution, containing 50 per cent ethanol and an equivalent amount of NaOH; basic solution 1:5 000, pH 7.5. — Table X.



The 1 per cent solution, completely colourless while fresh, gradually, even if kept in a refrigerator, becomes slightly yellowish red, and its activity is at the same time rapidly reduced. The reason for these changes is probably the hydrolytic decomposition of divaricatic acid (I) into divaric (II) and divaricatinic (III) acids (29) (see above).

Gyrophoric acid,



Gyrophoric acid and the following, its monomethyl ether, umbilicaric acid, belong to the so-called tridepsides; they have three

benzene rings bound together by depside bonds. In the majority of cases, they are present in the same lichens.

Gyrophoric acid was extracted from two lichens, both of which probably contain gyrophoric acid only: Umbilicaria pustulata (collected from a rock in the neighbourhood of Helsinki 1949) and Ochrolechia tartarea (data and place of collection as above). The lichens were extracted with ether (Soxhlet) [*e.g.* Koller (41)], and the precipitate arising on evaporation was crystallised several times from acetone. The acid crystallises with great difficulty and in very small, slightly yellowish crystals, m.p. 198—200 °C (Zopf 202—203°, Asahina 220°C). Gyrophoric acid takes up one mol of water of crystallisation and surrenders it with fairly great difficulty; apparently the remaining water of crystallisation may account for the low melting point.

Titration: 115.6 mg of crystals (acetone, bromthymolblue); 2.50 ml of 0.1-n NaOH were consumed. As monobasic acid, the molecular weight is 462 (calculated M = 468 without water of crystallisation and 486 including one mol of water of crystallisation).

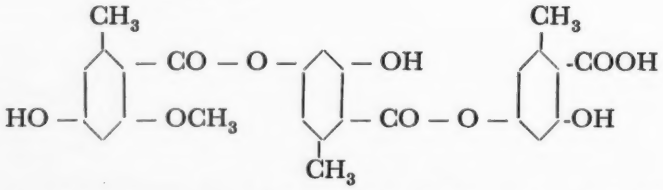
1 per cent aqueous solution, containing 50 per cent of alcohol and an equivalent amount of NaOH, gradually intensifying cherry red colour. Basic solution as usual, pH 7.5. — Table XI.

TABLE XI

THE DILUTION SERIES OF GYROPHORIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	+	++	++	++	++	++	++	++	++	++	++
Staph.	+	++	++	++	++	++	++	++	++	++	++
Streptoc.	−	+	(++)	++	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht.	−	+	++	++	++	++	++	++	++	++	++
Megath.	+	++	++	++	++	++	++	++	++	++	++
Subtilis ...	+−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Coli	+	++	++	++	++	++	++	++	++	++	++
Parat.	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

Umbilicaric acid, $C_{25}H_{22}O_{10}$



Preparation from Gyrophora vellea (collected from a moist rock in the neighbourhood of Helsinki 1949), according to Koller and Pfeiffer (44): the lichen was extracted with ether, the black tar-like substance arising in the extract being removed and the ether evaporated to dryness. The remainder was boiled for three hours in a water bath with alcohol to decompose the gyrophoric acid, and the evaporated precipitate was dissolved in ether. The ether solution was shaken together with $NaHCO_3$ solution, and the umbilicaric acid dissolved precipitated with hydrochloric acid. The precipitate was again shaken in ether, evaporated and precipitated from hot alcohol with boiling water: reddish powder, m.p. 183—184°C. (Hesse 185—186°, Zopf 189°, Koller and Pfeiffer 203°C.)

1 per cent aqueous solution, containing 25 per cent alcohol and an equivalent amount of NaOH, light yellow. Basic solution 1:5 000, pH 7.5. — Table XII.

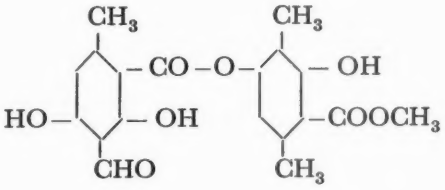
TABLE XII

THE DILUTION SERIES OF UMBILICARIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	++	++	++	++	++	++	++	++	++	++	++
Staph. ...	+	++	++	++	++	++	++	++	++	++	++
Streptoc.	–	++	++	++	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht. ...	+	++	++	++	++	++	++	++	++	++	++
Megath.	++	++	++	++	++	++	++	++	++	++	++
Subtilis ...	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$	++$^+$
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

The Depsides of the β-Orcinol Group

Atranorin, $C_{19}H_{18}O_8$



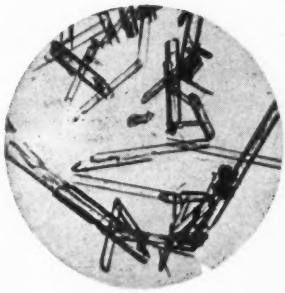


Fig. 8. Crystals of atranorin from acetone, × 50.

Preparation from Stereocaulon paschale (collected at Laukaa 1949), mainly according to Zopf (96): the lichen was extracted with ether for 48 hours (Soxhlet), evaporated down to a small amount, dissolved in hot chloroform, evaporated, and precipitated with alcohol, crystallised from acetone: slightly reddish needles, m.p. 190–193°C (Asahina 194–5°, Zopf 195–7°C). — Fig. 8.

Titration: 112.4 mg of crystals (ethanol, phenolphthalein); 6.1 ml

TABLE XIII

THE DILUTION SERIES OF ATRANORIN, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	–	++	++	++	++	++	++	++	++	++	++
Staph.	–	++	++	++	++	++	++	++	++	++	++
Streptoc.	–	++	++	++	++	++	++	++	++	++	++
Pneumoc.	+	++	++	++	++	++	++	++	++	++	++
Dipht.	–	++	++	++	++	++	++	++	++	++	++
Megath.	–	–	++	++	++	++	++	++	++	++	++
Subtilis ...	–	++⁻	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺	++⁺
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat.	+	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

of 0.1-n NaOH were consumed. As dibasic acid, molecular weight is 368 (calculated M = 374).

1 per cent aqueous solution, containing 40 per cent of alcohol and an equivalent amount of NaOH. Fresh, the solution is bright yellow but will soon turn darker, and after 4—5 days is dark violet-brown. Basic solution as usual, pH 7.5. — Table XIII. — Due to the very poor solubility of atranorin it is not possible to study the activity of free acid by means of dilution series.

Fig. 9. Crystals of atranol from water, × 50.

Atranol, $C_8H_8O_3$

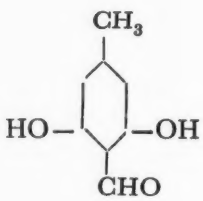


TABLE XIV

THE DILUTION SERIES OF ATRANOL, BEGINNING FROM 1:2 500, FOR KEY, SEE P. 40

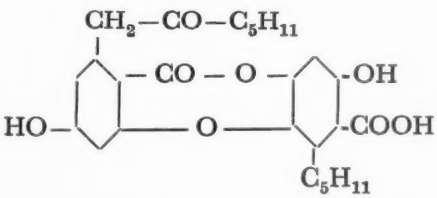
	1:2 500	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	Control	Control
Sarcina ...	−	−	−	−	+	++	++	++	++	++	++
Staph. ...	−	−	−	++	++	++	++	++	++	++	++
Streptoc.	−	−	−	++	++	++	++	++	++	++	++
Pneumoc.	−	++	++	++	++	++	++	++	++	++	++
Dipht. ...	−	−	−	−	++	++	++	++	++	++	++
Megath.	−	−	−	++	++	++	++	++	++	++	++
Subtilis ...	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Coli	−	±	++	++	++	++	++	++	++	++	++
Parat. ...	−	−	++	++	++	++	++	++	++	++	++
Proteus ...	−	−	−	++	++	++	++	++	++	++	++
Pseudom.	(+)	++	++	++	++	++	++	++	++	++	++

Atranol was prepared in order to explain the inhibitory effect on proteus growth, seen on lichen pieces in the preliminary tests, but not in the atranorin isolated from the active lichen (Stereocaulon paschale). The preparation was effected in the Chemical Laboratory of the Institute of Technology from crystalline atranorin according to Pfau (61): atranorin was boiled in ten times the quantity of glacial acetic acid in an oil bath at 140—150°C for ten hours. The glacial acetic acid was evaporated in a vacuum, and the atranol separated from β-orcinol carbonic acid by means of 10 per cent $NaHCO_3$-solution and crystallised from water. Yellowish brown, needle-like crystals, m.p. 120—121°C (Pfau 124°C without water of crystallisation). — Fig. 9.

1 per cent solution in water by heating to boiling. Basic solution 1:2 500, pH 7.5. — Table XIV.

The Depsidones of the Orcinol Group

Physodic acid, $C_{26}H_{30}O_8$



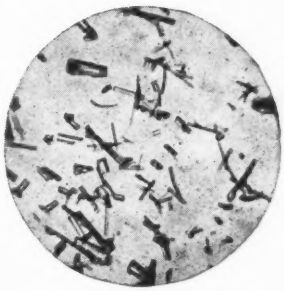
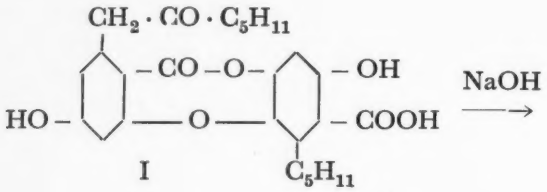


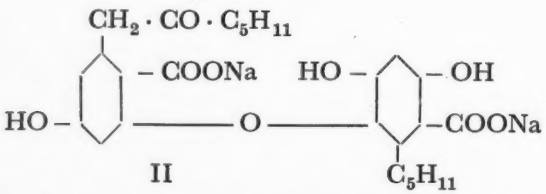
Fig. 10. Crystals of physodic acid from methanol, × 300.

The acid was isolated from Parmelia physodes (collected from birch rind at Laukaa 1948), essentially according to Asahina and Nogami (6): the lichen was extracted for three days with ether (Soxhlet). The precipitate arising on the evaporation of the ether (atranorin) was filtered off and the ether evaporated to dryness. The remainder was washed with chloroform, and crystallised several times from methanol: pure white microcrystals, m.p. 195°C (Asahina: 205°, Zopf: 200—202°, Hesse: 190—192°C). — Fig. 10.

Titration: 100.0 mg crystals (asetone, phenolphthalein); 4.22 ml of 0.1-n NaOH were consumed. As a dibasic acid, M = 471 (calculated M = 470).

The dibasic character of the physodic acid is due to the fact that the lactonic ring contained in it opens even at room temperature when titrated with alkali, physodic acid (I) changing into isophysodic acid (II) (6):





1 per cent aqueous solution, containing 25 per cent of alcohol and an equivalent amount of NaOH, light cherry-red, becomes gelatinous at approx. +5°C but rapidly turns fluid again at room temperature. Basic solution 1:5 000, pH 7.5. — Table XV.

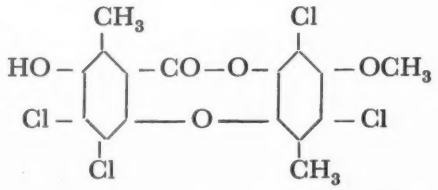
TABLE XV

THE DILUTION SERIES OF PHYSODIC ACID, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	–	–	–	–	–	(++)	++	++	++	++	++
Staph.	–	–	–	(+)	++	++	++	++	++	++	++
Streptoc.	–	–	–	–	(++)	++	++	++	++	++	++
Pneumoc.	–	–	–	–	++	++	++	++	++	++	++
Dipht.	–	–	–	–	–	–	++	++	++	++	++
Megath.	–	–	–	–	(+)	++	++	++	++	++	++
Subtilis ...	–	–	–	–	+–	+++	+++	+++	+++	+++	+++
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat.	++	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

Also a 1/2 per cent emulsion was made of the physodic acid by dissolving the acid in ethanol and adding this, by dropping, into hot water. With a test series made from the emulsion in neutral nutrient medium the same results were attained as with the acid titrated with base.

Diploicin, $C_{16}H_{10}O_5Cl_4$



Diploicin is one of the three lichen substances containing chlorine. The diploicin employed in these tests was received from Dr. V. C. Barry in Dublin (see p. 37); it was isolated in England from Buellia canescens and crystallised here from alcohol, once: small white needles, containing alcohol of crystallisation; the substance is decomposed at 202—204°C, finally melts at 212—215°C. (Nolan *et al.* (54): 232°C without alcohol of crystallisation.)

TABLE XVI

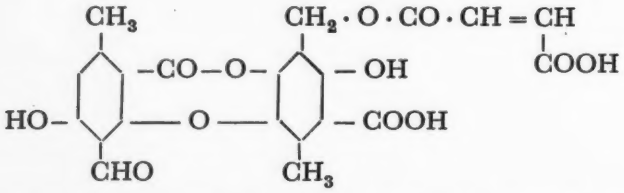
THE DILUTION SERIES OF DIPLOICIN, FOR KEY, SEE P. 40

	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	Control	Control
Sarcina ...	–	–	–	–	–	+	++	++	++	++	++
Staph. ...	–	–	–	–	+	++	++	++	++	++	++
Streptoc.	–	–	–	–	–	++	++	++	++	++	++
Pneumoc.	–	–	–	++	++	++	++	++	++	++	++
Dipht. ...	–	–	–	–	–	+	++	++	++	++	++
Megath.	–	–	–	–	–	–	–	+	++	++	++
Subtilis ...	–	–	–	–	–	–	++⁻	++⁻	++⁺	++⁺	++⁺
Coli	++	++	++	++	++	++	++	++	++	++	++
Parat. ...	+	++	++	++	++	++	++	++	++	++	++
Proteus ...	++	++	++	++	++	++	++	++	++	++	++
Pseudom.	++	++	++	++	++	++	++	++	++	++	++

1 per cent aqueous solution was made, containing 40 per cent of alcohol and an excess amount of NaOH, colourless, rather stringy solution. Basic solution 1:5 000, pH 7.5. When dissolved, the depside ring of diploicin opens like that of physodic acid [Barry (11)].

The Depsidones of β-Orcinol Group

Fumarprotocetraric acid, $C_{22}H_{16}O_{12}$



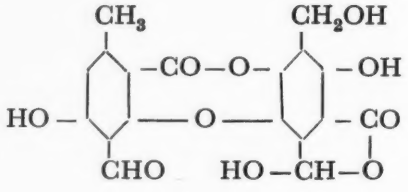
Preparation from Cetraria islandica (collected from rocks on the Island of Degerö 1949), essentially according to Zopf (96) and Asahina and Tanase (8): after extraction with ether (p. 00) the lichen was extracted with acetone (Soxhlet), evaporated to dryness and crystallised from acetone: fairly white mass of very small crystals, which do not melt but contract and turn red on heating; carbonise at 260—270°C.

Titration: 88.9 mg of crystals (acetone, bromthymol blue); 3.80 ml of 0.1-n NaOH were consumed. Molecular weight as dibasic acid 468 (calculated M = 472).

1 per cent aqueous solution, containing 50 per cent alcohol and an equivalent amount of NaOH, pale yellow. Basic solution 1:5 000, pH 7.5.

Fumarprotocetraric acid was not found to have any distinct growth-inhibiting effect on the bacteria studied.

Salazinic acid, $C_{18}H_{12}O_{10}$

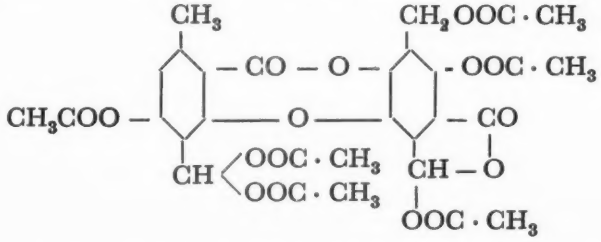


Preparation from Parmelia saxatilis (collected from stones at Laukaa 1948) essentially according to Zopf (96) and Asahina and Asano (4): the lichen was first extracted for 24 hours with ether, to dissolve the atranorin. Subsequently it was extracted with acetone for 8 hours and crystallised again from acetone, twice: small crystals of a slightly reddish tint, which, when filtered, form a paper-like mass. The crystals do not melt but gradually become red on heating and carbonise at 260—270°C.

Salazinic acid could not be titrated.

1 per cent aqueous solution in the same way as from fumarprotocetraric acid, deep crimson, pH 7.5.

Hexa-acetate of salazinic acid $C_{30}H_{26}O_{17}$



was prepared, according to Asahina and Asano (4), from salazinic acid to identify it: the salazinic acid was mixed with acetanhydride and a drop of concentrated sulphuric acid added, resulting in dissolution. The precipitate obtained by adding water was washed with water and crystallised twice from weak alcohol: white needle-like crystals, m.p. 175°C (Asahina 178°C).

1 per cent aqueous solution, containing 40 per cent of alcohol and 60 per cent of 0.1-n NaOH, yellow, gradually grows beautifully orange-red. Basic solution 1:5 000, pH 7.5.

Both the salazinic acid and its hexa-acetate proved in these tests to be inactive.

Discussion

No very far-reaching conclusions can be drawn from the tests made with the methods and nutrient media employed. For instance, the amount of bacteria present »in broth grown for 24 hours» varies according to the type of the broth, etc. Nor is the ordinary broth, employed exclusively in these tests, the best possible nutrient medium for Streptococcus and Pneumococcus in particular. On the other hand, the results obtained at different times are somewhat different from each other, due to the general character of biological phenomena.

Of the 20 lichen substances or their closely related compounds studied, the first four belong to the group of *aliphatic lichen substances*. They are all — though to greatly differing degrees — active. The d-protolichesteric acid, contained as such in the Iceland moss, and its isomer lichesteric acid, are almost completely similar in effect; the transfer of the double bond to the lactone ring, therefore, does not seem to change the behaviour of the acid towards bacteria (cf. fungus tests further below). On the other hand, d-lichesteric acid is obviously a more stable compound than its isomer; its effect was not found to weaken when kept as a solution, as does that of d-protolichesteric acid (p. 42). Lichesterylic acid is considerably weaker than the former, which is likely to be due, at least in part, to the opening of the lactone ring.

The inhibitory titres of d-protolichesteric and lichesteric acid on Gram-positive bacteria are remarkably high, whereas they only weaken the growth of certain Gram-negative rods at 1:5 000 (Parat., Prot., Pseudom., cf. the preliminary tests, p. 26). The inhibitory titres on diphtheria bacillus (1:160 000—1:320 000) are relatively the highest. The 1:80 000 for Staphylococcus corresponds to the value obtained by Cavallito *et. al.* (16) (p. 20).

Caperatic acid is much weaker than the two former, but seems similar in character: its inhibitory effect in these tests was apparent on Sarcina, Streptococcus and diphtheria bacillus only, with which other acids too have given the highest inhibitory values.

The effect of *pulvic acid derivatives*, the pinastric acid isolated from lichen and the synthesised anilide of pulvic acid, remains small. The strongest inhibitory effect was seen with the non-pathogenic

Sarcina, Megatherium and Subtilis. No effect was obtained on Gram-negative rods.

Usnic acid inhibits all Gram-positive bacteria (1:40 000—1:160 000) but has no effect at all on the Gram-negative ones. Decarbousnic and usnolic acid were inactive in these tests as well, a behaviour previously established by Marshak *et al.* (52) (p. 19) of both these substances towards TB, and by Shibata *et al.* (82) (p. 19) with usnolic acid.

An interesting group is that of the *depsides*. Of the orcinol-type depsides, divaricatic acid is strongly active and behaves towards the various bacteria almost completely similarly to usnic acid (p. 47, 51). Evernic acid, differing from divaricatic acid merely in its shorter side chain, is only weakly bacteriostatic; the activity, hence, seems to increase with the lengthening of the side chain. The effect of divaricatic acid is reduced when kept as an alkaline solution, obviously due to its being partially decomposed through hydrolysis (p. 52); evernic acid, when tested in the usual way (pH 7.5) gave no distinctly positive results at all. Dissolved without alkali and in neutral nutrient medium it proved weakly active. The tridepsides, gyrophoric and umbilicaric acids, and the depside of the β-orcinol group, atranorin, are also very weakly active. These acids have CH_3 groups only as side chains.

Among the *depsidones* the results seem to be similarly divided: the orcinol-type depsidone, physodic acid, is distinctly active, the β-orcinol-type fumarprotocetraric and salazinic acids and the hexa-acetate of the latter not at all. The effect of physodic acid is evidently not reduced by the opening of the lactone ring as the results obtained both when titrated with an alkali and as a free acid (pH $\leqq$ 7) are identical (p. 59).

A special position is held by the chlorine-containing depsidone, diploicin. It differs from the other lichen substances studied in its titre differences to the various bacteria being large, and is of a somewhat similar type in character, primarily to pinastric acid, much weaker than diploicin. The inhibitory titre 1:80 000 to diphtheria bacillus corresponds to that obtained by Barry (11) (p. 18). Among the lichen substances studied, diploicin has the strongest effect on Megatherium and Subtilis.

The schematic figures (p. 64) give a comparison between the

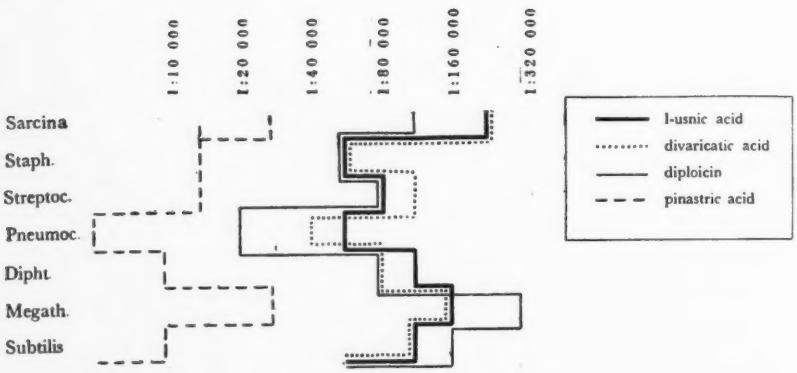


Fig. 11. The antibiotic activity of l-usnic, divaricatic and pinastric acids and of diploicin compared with each other. The diagram shows the minimum inhibitory titres of the substance for the different bacteria; if growth-weakening effect, however, has been observed in the dilution series the entry is shown half way between the titre values.

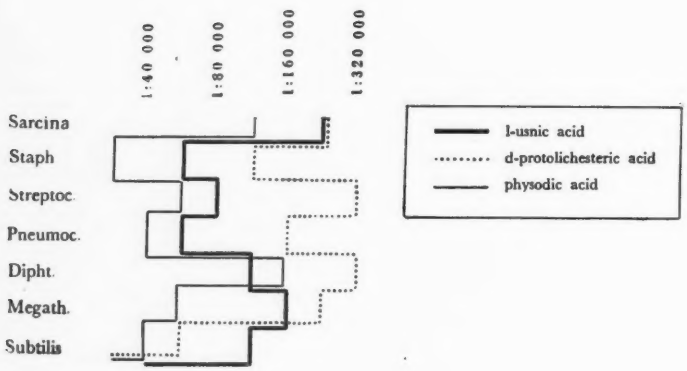


Fig. 12. The antibiotic activity of d-protolichesteric, l-usnic and physodic acids, key as above.

tests results obtained with active lichen substances of different types.

The hydrolysis product of atranorin, the single ringed atranol, differs decisively in its effect from the lichen substances studied, having a relatively much stronger effect on Gram-negative rods. Proteus is inhibited at 1:10 000, S. paratyphi at 1:5 000, E. coli at 1:2 500, at which the growth of Ps. aeruginosa also is very weak. The presence of atranol or other decomposition products of corresponding type in the lichen would, hence, well account for the inhibitory effects found in the preliminary tests on Gram-negative rods (see p. 35).

C. TESTS WITH TUBERCLE BACILLUS STRAINS

Methods

Dilution series of eighteen of the above-mentioned lichen substances or related substances were made into two TB strains (owing to the quantities available being scarce, decarbousnic acid and atranol had to be left unstudied). The strains employed were:

Mycob. tuberc. hominis 1943, ... an old laboratory strain
» » » 3857, ... a freshly isolated strain.

The cultures were made parallelly with two media:

Kirchner Modification:

$Na_2HPO_4 \cdot 2\ H_2O$	3.0
KH_2PO_4	4.0
$MgSO_4 \cdot 7\ H_2O$	0.6
Sodium citrate $\cdot 2\ H_2O$	2.5
Asparagine	5.0
Glycerin	20.0
Aq. dest.	1 000.0
Sterilised, then added bovine serum	100.0

Dubos's medium (19):

Asparagine	1.0
$Na_2HPO_4 \cdot 12\ H_2O$	6.3
KH_2PO_4	1.0
Sodium citrate $\cdot 2\ H_2O$	1.5
$MgSO_4 \cdot 7\ H_2O$	0.6
Ad. dest.	ad 1 000.0
Sterilised, then added bovine serum albumin (Powiet Producten, Amsterdam)	0.2 %
Tween 80	0.05%

The basic solutions were prepared in the same way as in the foregoing tests: 1:5 000, pH was ascertained to be approx. 7.0. 1 per cent emulsion was used for the evernic and divaricatic acid basic solution; pH of nutrient medium 7.0. A dilution series in negative powers of 4 was made (1:5 000, 1:20 000 etc.) of each substance. 4.5 cc of nutrient medium was measured into each tube. The series were inoculated with 0.1 cc of Dubos's culture grown for 6—8 days and diluted 1:20. Apart from certain control series carried out later, the Kirchner series and the Dubos series, respectively, were cultivated at the same time from one and the same dilution. The cultures were checked after 10 and 20 days. In the control series made, 1 per cent alcohol concentration was found to have no inhibitory effect on the growth of any of the series.

Results

The results obtained at different readings are entered only insofar as they differed mutually from the respective controls. ++ = growth similar to that in controls. + = growth weaker than in controls. — = no growth. — Table XVII.

TABLE XVII

THE ANTIBIOTIC EFFECT OF VARIOUS LICHEN SUBSTANCES AGAINST TWO TB-STRAINS

Substance	Nutrient medium	Mycobacterium tuberc. hominis 1943					Mycobacterium tuberc. hominis 3857				
		1:5 000	1:20 000	1:80 000	1:320 000	1:1 280 000	1:5 000	1:20 000	1:80 000	1:320 000	1:1 280 000
Caperatic acid	K	±+	++	++	++	++	+	++	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
d-Protolichest. acid	K	−	++	++	++	++	±+	++	++	++	++
	D	−	+	++	++	++	−	+	++	++	++
d-Lichesteric acid	K	−	++	++	++	++	−	++	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
Lichesterylic acid	K	−	+	++	++	++	−	++	++	++	++
	D	−	−	++	++	++	−	−	++	++	++
Pinastric acid	K	−	+	++	++	++	−	++	++	++	++
	D	−	−	++	++	++	−	−	++	++	++
Anilide of pulvic acid	K	−	−	++	++	++	−	+	++	++	++
	D	−	−	−+	+	++	−	−	−+	++	++
l-Usnic acid	K	−	−	+	++	++	−	−	+	++	++
	D	−	−	−	++	++	−	−	−	++	++
Usnolic acid	K	++	++	++	++	++	++	++	++	++	++
	D	++	++	++	++	++	++	++	++	++	++
Evernic acid	K	−	++	++	++	++	−	++	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
Divaricatic acid	K	−+	++	++	++	++	−+	++	++	++	++
	D	−	±+	++	++	++	−	++	++	++	++
Gyrophoric acid	K	−	++	++	++	++	−	++	++	++	++
	D	−	−	++	++	++	−	−	++	++	++
Umbilicaric acid	K	++	++	++	++	++	++	++	++	++	++
	D	++	++	++	++	++	++	++	++	++	++
Atranorin	K	−	+	++	++	++	−	+	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
Physodic acid	K	?	++	++	++	++	?	++	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
Diploicin	K	−±	±+	++	++	++	−	++	++	++	++
	D	−	++	++	++	++	−	++	++	++	++
Fumarprotoc. acid	K	++	++	++	++	++	++	++	++	++	++
	D	++	++	++	++	++	++	++	++	++	++
Salazinic acid	K	++	++	++	++	++	++	++	++	++	++
	D	++	++	++	++	++	++	++	++	++	++
Acetate of salaz. acid	K	++	++	++	++	++	++	++	++	++	++
	D	++	++	++	++	++	++	++	++	++	++

Discussion

The fact that the titres obtained, generally, are lower than those quoted in literature for the same substances, particularly usnic acid, is likely to be due to the large inoculum used. Inoculated, mainly, from one and the same dilution, the series made, on the other hand, are well comparable mutually. The inhibitory titres obtained by Dubos's nutrient medium, as a rule, are somewhat higher, contrary to the report by Bustinza and Lopez (15) (cf. p. 19). The »old» and the »fresh» strain employed in the tests yielded almost completely identical results.

Of the acids studied, thirteen revealed a distinctly inhibitory effect on the growth of TB; five lichen substances proved to be inactive: usnolic, fumarprotocetraric and salazinic acid and the hexa-acetate of salazinic acid found inactive in previous tests also, and in addition umbilicaric acid.

The inhibitory titres of *aliphatic lichen substances* vary between 1:5 000 and 1:20 000. Caperatic acid is the weakest, as it was in the tests made with rapidly growing bacteria, whereas lichesterylic acid seems the most effective, in marked contrast to those tests. Protolichesteric acid is somewhat weaker than lichesteric acid, which may be due to the lengthy growth period (see p. 42).

The *pulvic acid derivatives* seem to affect Mycob. tuberculosis relatively more strongly than the rapidly growing bacteria: the anilide of pulvic acid is found to have a growth-reducing effect on one of the strains up to a titre of 1:320 000, growth being completely arrested at 1:20 000 only.

The titres obtained for l-*usnic acid*, 1:20 000—1:80 000, correspond most closely to the values obtained by Marshak (49) (cf. p. 18). Among the acids studied usnic acid remains the best against the tubercle bacillus.

The results obtained with the *depsides* differ considerably from those obtained with rapidly growing bacteria: the order obtained, according to activity, is almost reversed. Evernic acid and the depside of the β-orcinol group, atranorin, are more effective than divaricatic acid, and the effect of gyrophoric acid extends one tube farther than that of other depsides, thus inhibiting the growth of the TB in a much higher titre (1:20 000) than that of any bacterium

studied previously. It is of interest that gyrophoric acid and its monomethyl ether, umbilicaric acid, which in the previous tests were roughly of the same value, when studied on the tubercle bacillus differ decisively (the same applies to yeast, p. 71).

In Dubos cultures, physodic acid inhibits growth in 1:5 000. In the Kirchner series, the 1:5 000 tube assumes a milky turbidity, and the growth is therefore difficult to judge. The titre for diploicin remained in these tests at 1:5 000, hence considerably lower than the value obtained by Barry (11) (p. 18). The main reasons for this are probably technical differences and different strains, particularly as the diphtheria bacillus titres of diploicin were in complete agreement (p. 63).

D. TESTS WITH DIFFERENT FUNGAL STRAINS

Tests with Yeast Fungus

With the broth solutions of 1:5 000 of the isolated lichen substances prepared previously, tests were made on the yeast strain Candida tropicalis (an old strain from collections). To ascertain the possible inhibitory effect of the alcohol employed in the dissolving, a control series was made beginning with a 4 per cent concentration. — Table XVIII:

TABLE XVIII

THE EFFECT OF ALCOHOL ON THE GROWTH OF YEAST FUNGUS

Alcohol conc. (per cent)	4	2	1	1/2	1/4	1/8	Controls	
2 days growth	+	++	++	++	++	++	++	++

The alcohol concentration of 1 per cent, thus, had no inhibitory effect on the yeast strain studied.

In the test series the pH of the nutrient medium was ascertained to be 7.0. The inoculation was made from a broth culture grown at

room temperature for two days, by dilution with physiological saline solution 1:10; amount of inoculum 0.1 ml Two tubes of 1.0 ml each of each lichen substance to be studied were available. The cultures were allowed to grow at room temperature, and were checked after 2 and 4 days.

The majority of the lichen substances were found to have no effect at all on the growth of the yeast fungus (Group I, table XIX). With a number of them inhibition or weakening of growth was visible after a growth period of 2 days, but no distinct difference was visible at the 4th-day check (Group II). With three lichen substances only was a complete inhibition of growth achieved after 4 days (Group III). When necessary, the results were checked microscopically.

TABLE XIX

THE EFFECT OF VARIOUS LICHEN SUBSTANCES ON THE GROWTH OF YEAST FUNGUS

Group I Inactive	Group II Retarded or weakened the growth	Group III Inhibitory effect
11 substances	4 substances	3 substances
d-Protolichesteric acid	Caperatic acid	d-Lichesteric acid
Lichesterylic acid	Divaricatic acid	l-Usnic acid
Pinastric acid	Atranorin	Gyrophoric acid
Anilide of pulvic acid	Diploicin	
Usnolic acid		
Evernic acid		
Umbilicaric acid		
Physodic acid		
Fumarprotocetraric acid		
Salazinic acid		
Hexa-acetate of salazinic acid		

Dilution series from the substances of Group III were made, employing the same method as above for the rapidly growing bacteria, and with the amount of inoculum as above. The series were checked after 1, 2 and 4 days. — Table XX.

TABLE XX

THE EFFECT OF THREE LICHEN SUBSTANCES ON THE GROWTH OF YEAST FUNGUS

Substance	Day	1:5 000	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	Control	Control
d-Lichesteric acid	1	−	−	−	+	+	++	++	++
	2	−	−	+	++	++	+++	+++	+++
	4	−	±	+	++	++	+++	+++	+++
l-Usnic acid	1	−	−	++	++	++	++	++	++
	2	−	−	+++	+++	+++	+++	+++	+++
	4	−	++	+++	+++	+++	+++	+++	+++
Gyrophoric acid	1	−	−	+	++	++	++	++	++
	2	−	+	+++	+++	+++	+++	+++	+++
	4	−	+	+++	+++	+++	+++	+++	+++

Of interest in the results are the decisive differences between d-protolichesteric and d-lichesteric acids, on the one hand, and the gyrophoric and umbilicaric acids on the other. (The difference between the effects of the latter had already emerged distinctly in the tests made with tubercle bacilli.) The pulvic acid derivatives proved to be inactive towards the yeast fungus.

Tests with Other Fungi

In addition, the effect of lichen substances on five further fungal strains was studied preliminarily:

Trichophyton (Ctenomyces) interdigitalis ..... an old strain from the collections

Trichophyton violaceum, pleomorphic form ..... » » »

Acorion gallinae ..... an old zoopathogenic strain isolated from black cock

Saprophlegia sp. ignota ..... an old strain isolated from the spawn of fish

Fomes annosus ..... an old zoo- and phytopathogenic strain

TABLE XXI

THE EFFECT OF VARIOUS LICHEN SUBSTANCES ON THE GROWTH OF DIFFERENT FUNGAL STRAINS

Only positive results in the titres 1:5 000—1:10 000 are entered. + indicates complete inhibition of the growth, (+) = growth retarded; the respective concentrations are given against the indicators.

	Candida tropicalis	Trichophyton (Ctenomyces) interdigitalis	Trichophyton violaceum	Acorion gallinae	Saprophlegia sp. ignota	Fomes annosus
Caperatic acid	(+) 1:5 000	(+) 1:10 000	(+) 1:10 000	(+) 1:10 000	(+) 1:10 000	(+) 1:10 000
d-Protolichest. acid						
d-Lichesteric acid	+ 1:5 000	(+) 1:10 000	(+) 1: 5 000		+ 1: 5 000	(+) 1: 5 000
Lichesterylic acid						
Pinastric acid						
Anilide of pulvic acid		+ 1: 5 000				
l-Usnic acid...	+ 1:5 000	+ 1:10 000	(+) 1:10 000	(+) 1:10 000	(+) 1:10 000	+ 1:10 000
Usnolic acid						
Evernic acid		+ 1: 5 000			(+) 1: 5 000	
Divaricatic acid	(+) 1:5 000	+ 1: 5 000	(+) 1:10 000	(+) 1: 5 000		+ 1: 5 000
Gyrophoric acid	+ 1:5 000	(+) 1: 5 000			(+) 1: 5 000	
Umbilicaric acid						
Atranorin ...	(+) 1:5 000	(+) 1:10 000			(+) 1: 5 000	
Physodic acid		(+) 1:10 000				
Diploicin	(+) 1:5 000	+ 1:10 000	+ 1: 5 000	(+) 1:10 000	+ 1: 5 000	+ 1:10 000
Fumarprotocetraric acid						
Salazinic acid						
Acetate of salaz.acid...						

Sabouraud's nutrient medium of the following composition was employed in the tests:

peptone	10.0
maltose	40.0
agar	20.0
aq. dest	1 000.0

An addition of each lichen substance was made into the nutrient medium warmed until fluid, to produce solutions of 1:5 000 and 1:10 000, pH approx. 7.0, which were allowed to set in an oblique position in the test tubes. The inoculation was effected by introducing, with a platinum wire loop, some of the fungus growing in the Sabouraud tube to three different spots in each tube. The culture grew at room temperature, and readings were taken after 5 and 10 days. (Table XXI, in which the results obtained with Candida tropicalis are also entered).

The results are rather compatible with those attained with the yeast: all substances inhibiting the growth of Candida tropicalis proved to be more or less active also against some of the other fungal strains studied. In addition, positive results were attained by evernic and physodic acids and with the anilide of pulvic acid. The difference observed in the yeast series of d-protolichesteric and lichesteric acid also emerges distinctly in these tests. According to these preliminary studies, the most effective substances are l-usnic acid and diploicin, but caperatic, d-lichesteric and divaricatic acids also display growth-inhibiting effects on the fungal strains studied. Eight substances are completely inactive; indeed, the usnolic acid, in particular, included in them, seems to possess qualities stimulating fungal growth.

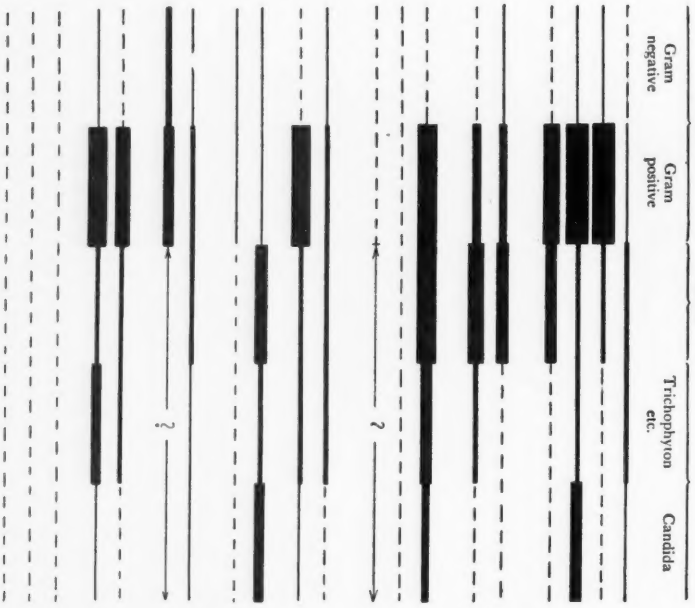


Fig. 13. Approximate diagram of the activity of the lichen substances studied on the different groups of micro-organisms. 0.5 mm in the width of the ribbon corresponds to an inhibitory titre of 1:5 000, 1.0 mm to 1:10 000, 1.5 mm 1:20 000 etc. The thin line indicates some weak activity established, ---- = no effect. In the group of rapidly growing bacteria, mean values of the inhibitory titres for the different bacteria have been calculated.

Discussion

In the preliminary tests carried out with pieces of lichens, 75 of the 149 forms studied, or 50 per cent, were found to be distinctly active, and 21 weakly or indefinitely active; including the latter, the percentage of active forms is 64. (Burkholder found 52 per cent from the American, and Stoll *et al.* 65.5 per cent from the Swiss species to be active, p. 18). Of the distinctly active forms, the activity of 50, or two-thirds, can be explained by known active compounds and our present knowledge of the lichen substances contained in lichens. Nothing definite can be said of the active components of the remaining one-third. The Gram-positive rods were in general susceptible, but the Gram-negative rods resistant. Sometimes there was a certain effect also against the Gram-negative rods. This effect was likely to be due, in part at least, to decomposition results of the lichen substances (p. 35, 65).

The total of crystalline lichen substances or related compounds studied amounts to 20, of which 13 were isolated from lichens as such, 6 derived from the isolated acids, and one was synthesised. The effects of these compounds on the different micro-organisms are schematically presented in Fig. 13. Due to the limited material available, the study of decarbousnic acid and atranol has been restricted to the group of rapidly growing bacteria only.

According to the schematic illustration, the main effect of lichen substances is on the rapidly growing Gram-positive bacteria and Mycobacterium tuberculosis. (It must be taken into consideration that the TB culture has been effected with relatively larger inocula than that of the rapidly growing bacteria, and hence the schema

may give a misleading picture on this point: in other papers titre values ranging from 1:600 000 to 1:1 000 000 have been obtained, *e.g.*, for d-protolichesteric acid and d-lichesteric acid against human TB (p. 20), and similarly, for usnic acid, values considerably exceeding those arrived at in the present study (p. 19); the titres obtained for rapidly growing bacteria, on the other hand, have generally been fairly compatible.) Some of the substances affecting the tubercle bacillus inhibit the growth of various fungal strains, some of these again that of the yeast fungus studied. Considerable activity against Gram-negative bacteria was displayed only by the hydrolytic product of atranorin, atranol.

Among the substances affecting *Gram-positive cocci and rods,* the most effective in these tests proved to be: d-protolichesteric and d-lichesteric acids, inhibiting Staphyloc. aureus at 1:80 000—1:160 000 and Corynebact. diphteriae mitis at 1:160 000—1:320 000; l-usnic acid and divaricatic acid with inhibitory profiles of nearly identical shape and with slightly lower titres; and the chlorine-containing diploicin, which seems to be most effective against dust bacteria.

The growth of *tubercle bacillus* was most actively inhibited by usnic acid (1:80 000), by the synthesised anilide of pulvic acid (complete inhibition at 1:20 000, growth-weakening effect at 1:320 000), by aliphatic lichesterylic acid and by gyrophoric acid of tridepside type (1:20 000).

Capable of inhibiting the growth of *Candida tropicalis,* in the concentrations tested, were only d-lichesteric, l-usnic and gyrophoric acids (1:5 000—1:10 000), while l-usnic acid and diploicin (1:5 000—1:10 000) best inhibited the growth of *other fungi.*

The analogy between the chemical structure of a substance and its bacteriostatic or fungistatic effect presents a complicated problem. The only definite inactive structural group seems to consist of the depsidones of β-orcinol type (3 of them studied). Usnic acid derivatives (2 of them studied) have also proved to be completely inactive, a result previously achieved also by Shibata *et al.* and Marshak *et al.* (p. 19) with several usnic acid derivatives. On the other hand, the most active effects on the groups of micro-organisms studied and their connection with the various structural formulae are difficult of explanation. However, certain features shared by

them can be seen: *e.g.* both the pulvic acid derivatives seem to possess specific affinity to the tubercle bacillus. But in many cases apparently small differences change the physiological effects of a substance very considerably. For instance, gyrophoric acid is distinctly active towards the tubercle bacillus and to some extent towards the fungi, whereas its monomethyl ether, umbilicaric acid, is completely inactive towards both of these groups. Similarly, in the isomeric d-protolichesteric and d-lichesteric acids, the location of the double bond obviously determines the activity of the substances towards fungi: when it moves into the lactone ring the substance becomes active towards fungi. This change does not alter the behaviour of the substance towards bacteria. *)

It seems that the activity of the lichen substances studied is dependent, simultaneously, on the structural formula type of the compound and — to a surprisingly great extent — on even seemingly insignificant details in the molecule, without any one of these factors alone ensuring antibiotic qualities to the substance. Judging from the several types of inhibitory profiles, the mechanisms of antibiotic action obviously are nearly as numerous as the active substances, and the present study in no way covers their elucidation. But, even on the basis of the present limited work, there is good reason to expect further surprises from the combination of different mechanisms — such as Marshak's streptomycin + usnic acid.

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* In the works by Shibata and Miura (p. 21) referred to above, those found to be the most effective against staphyloc. and an avian TB strain were protolichesteric acid with its derivatives, usnic acid, didymic acid with its related compounds, and the orcinol-type depsides and depsidones; lichen substances of aliphatic fatty acid type and the depsides and depsidones of β-orcinol type were found weakly active only towards the bacteria studied by the author. Hence the results correspond with those obtained in the present work with rapidly growing bacteria.

Summary

In preliminary tests made with pieces of lichen, 75 out of 149 forms (50 per cent) were found distinctly active towards a minumum of two bacteria studied. Of these, the active substance of 50, or two-thirds, was known. Gram-positive bacteria only, as a rule, were susceptible; the distinct inhibitory effect on Gram-negative rods observed in some cases was obviously due to the decomposition products of lichen substances.

Of the total of 20 crystalline lichen substances or related compounds 15, of different inhibitory profiles, proved to be more or less active against the rapidly growing Gram-positive bacteria and the tubercle bacillus. The substances tested represented eight types of lichen substances: the *aliphatic lactones* (d-protolichesteric and d-lichesteric acids) inhibited fairly strongly the growth of rapidly growing bacteria in particular, those of the *aliphatic fatty acid type* (lichesterylic and caperatic acids) revealing a comparatively better inhibitory effect on the growth of the TB, as did the *pulvic acid derivatives* (pinastric acid and the anilide of pulvic acid). The *cumarone derivative* (usnic acid) was of the same effect range as the most active lichen substances of other types. The activity of the *depsides of orcinol type* (evernic, divaricatic, gyrophoric and umbilicaric acids) and that of the *depsidones of orcinol type* (physodic acid) seemed to increase with the growth in length of the side chains, except as regards TB. The chlorine-containing diploicin was comparatively best in effecting the Gram-positive dust bacteria. Two usnic acid derivatives only (usnolic and decarbousnic acids), and the *depsidones of β-orcinol type* (fumarprotocetraric and salazinic acids and the hexa-acetate of salazinic acid) were found completely inactive. The *depside of β-orcinol type* (atranorin) also was very weakly active only against the rapidly

growing bacteria, inhibiting the growth of the TB comparatively better.

The decomposition product of atranorin (atranol) had a distinct inhibitory effect on the growth of Gram-negative bacteria.

With some individual lichen substances of different types distinct activity on various fungal strains was observed.

The nature of the antibiotic activity characteristic of the different types of lichen substances seems to depend, apart from on the basic structural formula of the substance, to a surprisingly great degree also on seemingly insignificant changes in their molecules.

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